1987

Gel electrophoresis, phase microscopy and water-holding capacity studies of beef sternomandibularis muscle treated with sodium chloride and alkaline phosphates

Bruce Colin Paterson
Iowa State University

Order Number 8805123

Gel electrophoresis, phase microscopy and water-holding capacity studies of beef *sternomandibularis* muscle treated with sodium chloride and alkaline phosphates

Paterson, Bruce Colin, Ph.D.

Iowa State University, 1987

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark __✓__.

1. Glossy photographs or pages ✓
2. Colored illustrations, paper or print _____
3. Photographs with dark background _____
4. Illustrations are poor copy _____
5. Pages with black marks, not original copy ✓
6. Print shows through as there is text on both sides of page _____
7. Indistinct, broken or small print on several pages _____
8. Print exceeds margin requirements _____
9. Tightly bound copy with print lost in spine _____
10. Computer printout pages with indistinct print _____
11. Page(s) _____ lacking when material received, and not available from school or author.
12. Page(s) _____ seem to be missing in numbering only as text follows.
13. Two pages numbered _____. Text follows.
14. Curling and wrinkled pages _____
15. Dissertation contains pages with print at a slant, filmed as received _____
16. Other _____

_____

_____

Gel electrophoresis, phase microscopy and water-holding capacity studies of beef sternomandibularis muscle treated with sodium chloride and alkaline phosphates

by

Bruce Colin Paterson

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Department: Animal Science
Major: Meat Science

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa
1987

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Fresh meat contains about 75% water, and the retention of this water, and added water, during storage and further processing is very important to the meat industry. The property of water retention in meat is important for several reasons. First, water retention is important for consumer satisfaction because moisture contributes to the color, flavor, juiciness and tenderness of meat products. Second, water retention is of economic importance because meat and meat products are sold by weight, and loss of water from these products will result in a monetary loss to the meat industry.

Forrest et al. (1975) defined water-holding capacity (WHC) as the ability of meat to retain water during the application of external forces involved in further processing. The WHC of a meat system is known to be affected by the addition of neutral salts such as sodium chloride (NaCl). The addition of NaCl increases meat product WHC by increasing the ionic strength of the meat system. This enhances electrostatic repulsions which causes a loosening of the myofibrillar structure and a swelling of meat tissue. This swelling allows more water to be held by the meat thereby increasing meat WHC (Hamm, 1960).

Phosphates are combined with NaCl and used in processed

meat products to reduce the amount of water lost from meat products. Hamm (1970) summarized the effect of phosphates on the increase in WHC in meat products as being due to: (a) an increase in pH of the product; (b) an increase in ionic strength of the product; (c) the ability to dissociate actomyosin, the main structural component of muscle, into actin and myosin, and (d) the ability to bind to meat proteins.

Trout and Schmidt (1984, 1986a) concluded that increases in ionic strength and pH were the two most important contributions of phosphates for improving the WHC of meat products. Lewis et al. (1986) reported that increases in meat system pH, and to a lesser extent, dissociation of actomyosin and general dispersion of myofibrillar proteins were the two most important phosphate contributions to increased meat WHC. Offer and Trinick (1983) studied the disruptive effect of NaCl and pyrophosphate (PP) on isolated rabbit myofibrils and theorized that changes in WHC of meat originated from changes in the volume of myofibrils. They attributed the observed changes in myofibril volume to the disruptive effect NaCl and PP had on transverse structural constraints, such as Z-lines, M-lines and actin-myosin crossbridges, that exist within the myofibril.

Offer and Trinick (1983) did not study the effect that

NaCl and PP had on titin and nebulin which are two large cytoskeletal proteins recently discovered by Wang et al. (1979) and Wang and Williamson (1980). Wang et al. (1984) proposed a model by which titin and nebulin form an elastic cytoskeletal framework with the thick and thin filaments of the myofibril. Therefore, titin and nebulin may play a vital role in regulating myofibril swelling and muscle tissue WHC. The primary objective of these studies was to use gel electrophoresis and phase-contrast microscopy to better understand how NaCl and phosphates, such as pyrophosphate, tripolyphosphate and sodium polyphosphate glassy, affect beef sternomandibularis muscle and relate any observed myofibril swelling or structural changes to changes in the WHC of this beef muscle.

LITERATURE REVIEW

Skeletal muscle structure

A review of skeletal muscle structure and its constituent myofibrillar proteins is important for an understanding of the action of salt and alkaline phosphates on myofibril swelling, myofibrillar protein extraction and muscle tissue water holding capacity. This treatise, unless otherwise noted, will deal with mammalian skeletal muscle.

Skeletal muscle fibers are held in an anatomical pattern by a series of continuous connective tissue sheaths. Whole muscle is surrrounded by a heavy connective tissue sheath called the epimysium. Anastomosing from the epimysium is the permysium which surrounds muscle bundles. Branching from the perimysium is the endomysium which surrounds single muscle fibers. The cell membrane of the skeletal muscle fiber, the sarcolemma, is closely associated with, but separate from, the endomysium (Romans et al., 1985).

Skeletal muscle fibers are long, cylindrical, unbranching cells with tapering ends. Each muscle fiber is multinucleated, formed by the fusion of mononucleated myoblasts during embryonic development. The length of a

muscle fiber normally ranges from 1 to 40 mm with an average length of 20 to 30 mm. Muscle fiber diameters characteristically range from 10 to 100 μm (Huxley, 1972). Myofibrils are the highly organized structures that serve as the contractile components of the muscle cell and comprise about 50% of total muscle protein. Myofibrils are elongated protein threads, 1 to 3 μm in diameter, lying parallel to one another with their long axis parallel to the long axis of the muscle fiber. Each myofibril extends along the entire length of the muscle fiber (Goll et al., 1984).

Huxley (1958, 1972) reported that myofibrils gave skeletal muscle its characteristic striated appearance when viewed with the phase contrast light microscope. The striated appearance is caused by the alternating patterns of the precisely aligned light and dark bands of adjacent myofibrils. The light regions in the phase contrast light microscope are weakly birefringent (isotropic), and these regions are termed the I-bands. Bisecting the I-bands is a narrow, dark line called the Z-line. The dark bands of the myofibril observed with the phase contrast microscope are strongly birefringent (anisotropic), and these bands are called the A-bands. A less dense zone of protein exists at the center of each A-band and is known as the H-zone. A narrow, dense band of protein, the M-line, is observed with electron microscopy in the center of the H-zone. The

distance from one Z-line to the next Z-line is called a sarcomere and is the repeating structural unit of the myofibril. The sarcomere length for resting muscle is 2.3 to 2.8 μm and 1.8 to 2.0 μm for contracting muscle.

Huxley (1972) viewed myofibrils with the electron microscope and described them as being composed of interdigitating thick and thin filaments. The thin filaments comprise the I-band and are 6 to 8 nm in diameter and 1.0 μm long. One end of the thin filament attaches to the Z-line while the other end extends into the region of the A-band between adjacent thick filaments. Thick filaments that compose the A-band region are 14 to 16 nm in diameter and 1.5 μm long. Unlike the I-band however, the thick filaments do not attach to the Z-line. Sarcomere lengths change during muscle contraction and relaxation, but the lengths of the thick and thin filaments do not change (Huxley, 1972).

Franzini-Armstrong (1970) observed two dense lines at each side of and parallel to the Z-line and named them the N-lines. The N_1-line (0.05 μm wide) is located 0.1 to 0.2 μm from the Z-line. Its position is fixed and will not change as sarcomere length changes. The N_2-line (0.15 μm wide) is located between the edge of the A-band and the N_1-line. In contrast to the N_1-line, the position of the N_2-line changes as sarcomere length changes. However, the

N_2-line keeps the same proportional distance from the Z-line and M-line when sarcomere length changes (Page, 1968).

Goll et al. (1984) reported that the proximate composition of mature mammalian skeletal muscle was as follows: water, 55-78%; protein, 15-22%; lipid, 2-20%; carbohydrate, 1-2%; ash, 1-2%; and nucleic acid (RNA, 100 mg/100 g; DNA, 25-30 mg/100 g). The approximate concentrations in living muscle of other important compounds are as follows: ATP, 5-15 mM; ADP, 0.5mM; phosphocreatine, 20 mM; creatine, 4-5 mM; carnosine, 350 mg/100 g; and anserine, 140 mg/100 g.

Skeletal muscle proteins have been classified into three groups according to their differing solubilities in salt solutions of various ionic strengths: sarcoplasmic, stromal and myofibrillar proteins (Szent-Gyorgi, 1960; Goll et al., 1984). Sarcoplasmic proteins make up 30-35% of the total muscle proteins and are soluble in neutral salt solutions having an ionic strength of less that 0.2. This fraction contains myoglobin and many of the enzymes associated with carbohydrate, lipid and amino acid metabolism. The stromal fraction is composed of those proteins that are insoluble in neutral aqueous solutions and contains primarily the connective tissue proteins collagen and elastin. This fraction comprises 10 to 15% of the total muscle protein (Goll et al., 1984).

Myofibrillar proteins constitute 50-56% of total muscle protein and are the largest class of muscle proteins. Generally, myofibrillar proteins are soluble in salt solutions that have ionic strengths above 0.4. However, after being removed from the myofibril some myofibrillar proteins are soluble in water. Thus, it is more appropriate to classify myofibrillar proteins as simply the proteins of the myofibril (Goll et al., 1984). A list of the more important myofibrillar proteins and their respective content as a percent of total myofibrillar protein is as follows: myosin, 45%; actin, 20%; tropomyosin, 5%; troponin, 5%; titin, 10%; nebulin, 5%; α-actinin, 2%; β-actinin, 1%; C-protein, 2%; M-protein (165 K protein), 3%; myomesin (185 K protein), <1%; creatin kinase, <1%; desmin, <1%; filamin, <1%; and, vinculin, <1% (Yates and Greaser, 1983).

Myofibrillar/cytoskeletal proteins

Myosin is the primary component of the thick filament and as such it serves an important role in the biological functioning of muscle and in meat quality. Myosin is a very large myofibrillar protein, with a molecular weight of about 470,000, and has six subunits. These six subunits consist of two heavy chains of about 200,000 to 223,000 daltons each and four light chains of approximately 16,000 to 21,000 daltons each (Yates and Greaser, 1983). The native myosin

molecule consists of a long, double stranded, almost totally α-helical, rod-like tail connected to two globular heads which are the sites of the enzymatic activity. The myosin molecule has an overall length of 170-175 nm with the rod portion being about 160 nm long and 2 nm in diameter and the two pear-shaped heads being about 19 nm long and 6.0 nm in diameter (Goll et al., 1984). Myosin has ATPase enzymatic activity, and the ability to bind actin and self-assemble into thick filaments *in vitro*. In the formation of thick filaments, the rod portions of adjacent myosin molecules aggregate to form the shaft of the thick filament while the globular heads bend out to form the actin-binding crossbridges (Goll et al., 1984). The size, function and solubility of myosin make it very important with respect to the meat quality factors of tenderness and water holding capacity (Lawrie, 1983).

Actin is the second most abundant myofibrillar protein and comprises 20% of the total myofibrillar protein (Yates and Greaser, 1983). Globular or G-actin is a single polypeptide chain with a molecular weight of approximately 42,000. One G-actin molecule contains one ATP molecule and one Ca^{2+} ion and, in the presence of 0.1 M KCl or 1 mM Mg^{2+}, polymerizes *in vitro* to form the double helical fibrous or F-actin polymer. F-actin has the ability to bind myosin and to modify the Mg^{2+}-ATPase activity of myosin. Also, actin

forms the backbone of thin filaments and interacts with tropomyosin and troponin. The Ca^{2+}-dependent interaction of actin and myosin in skeletal muscle is controlled by the tropomyosin/troponin regulatory complex (Goll et al., 1984).

Tropomyosin is a long, rod-shaped protein molecule that constitutes 5% of the myofibrillar proteins and is part of the regulatory system of contraction. It is located in each of two grooves of the F-actin helix, associating end to end forming two strands of tropomyosin running the entire length of the thin filament (Goll et al., 1984). The tropomyosin molecule is composed of two polypeptide chains, α-tropomyosin (33,300) and β-tropomyosin (33,000). These subunits are almost entirely α-helical and lie in register in a two-stranded, coiled-coil arrangement (Mak et al., 1980).

Troponin is a globular protein with a molecular weight of about 69,000, and it makes up 5% of the total myofibrillar protein. Troponin and tropomyosin, together, are responsible for the regulation of muscle contraction via calcium levels. As determined by SDS-PAGE, native troponin consists of three subunits: troponin-T (TN-T), troponin-I (TN-I) and troponin-C (TN-C) which have molecular weights of approximately 37,000, 24,000 and 18,000, respectively (Goll et al., 1984). TN-T attaches the troponin proteins to tropomyosin. TN-I inhibits actomyosin ATPase by inhibiting

the actin-myosin interaction. TN-C is capable of reversibly binding Ca^{2+} ions. Huxley (1972) proposed that the troponin - tropomyosin complex controls contraction via the steric blocking model. In this model, when the Ca^{2+} concentration is below 10^{-8} M, tropomyosin is located between actin in the thin filament and the myosin crossbridge. As the calcium concentration rises after its release from the sarcoplasmic reticulum, TN-C binds the Ca^{2+} ions. The binding initiates a series of conformational changes in the thin filament and ultimately results in the tropomyosin strand being shifted, allowing myosin crossbridges to interact with actin in the thin filament.

Immunofluorescence studies have shown that α-actinin is associated with actin and is the major protein located in the Z-line (Suzuki et al., 1976). Alpha-actinin has a molecular weight of 200,000 and is composed of two subunits of 100,000 daltons each (Robson et al., 1970). The molecule constitutes 2% of the myofibrillar protein and is about 3.5 nm wide and 40 nm long (Yates and Greaser, 1983; Suzuki et al., 1976). It has been proposed that in vivo, α-actinin has a structural role of attaching thin filaments of opposing sarcomeres at the Z-line (Yamaguchi et al., 1983).

Desmin was first isolated from smooth muscle (Huiatt et al., 1980) and then from skeletal muscle (O'Shea et al., 1981). Desmin has a molecular weight of 55,000 and

represents less than 1% of the total myofibrillar protein. Desmin has the ability to self-assemble into long, 10 nm diameter "intermediate" filaments in vitro (Huiatt et al., 1980). Immunoelectron microscope localization evidence suggests that desmin 10 nm intermediate filaments link adjacent myofibrils together at the Z-line level (Huiatt et al., 1980; O'Shea et al., 1981). Desmin may be important in meat quality because it disappears during postmortem aging (Robson and Huiatt, 1983).

C-protein is a constituent of the thick filament of the myofibril. It has a molecular weight of 140,000 (Starr and Offer, 1978) and it represents 2% of the total myofibrillar protein (Goll et al., 1984). There are seven bands, 43 nm apart, of C-protein on each side of the M-line on the thick filament (Craig and Offer, 1976). Various functions have been suggested for C-protein such as effecting the actin - myosin interaction, influencing myosin crossbridge movement and maintaining the thick filament shape during muscle contraction (Starr and Offer, 1978; Moos, 1981).

M-line proteins make up less than 3% of the myofibrillar proteins and are associated with the thick filament (Goll et al., 1984). The M-line of thick filaments is composed of three proteins, namely M-protein (165 K protein), myomesin (185 K protein) and creatine kinase (two subunits, 42,000 each) (Trinick and Lowey, 1977; Grove and

Eppenberger, 1983). When longitudinal sections of muscle are examined by electron microscopy, the M-line appears as 3 to 5 lines perpendicular to the long axis of the thick filament. Trinick and Lowey (1977) proposed that the M-line functions to hold the thick filaments in their proper three-dimensional register.

Huxley and Hanson (1954) observed that, when myofibrils were treated with salt (NaCl) solutions to remove thick or thin filaments, the myofibrils did not fall apart. Instead, myofibrils remained together and elastic. They proposed that a set of very fine elastic filaments provided continuity in the sarcomere even after removal of the thick and thin filaments. Sjostrand (1962) stretched frog muscle to a point where a gap developed between the A-band and I-band filaments. Fine filaments were observed bridging this gap. The thickness of those filaments measured 30 A or less compared with 70 A for actin filaments. Sjostrand (1962) termed them "gap" filaments and proposed that the ends of the thick and thin filaments were linked by these gap filaments. Carlsen et al. (1965) also reported observing gap filaments in overly stretched rabbit muscle fibers.

Locker and Leet (1975) reported that when bovine sternomandibularis muscle was stretched to five times its original rest length, a gap occurred between the A- and

I-bands. The gap was spanned by gap filaments which appeared to be continuous with the thick filaments. Other observations included that during stretching, the A-band would become broader due to stretching and dislocation of thick filaments, half sliding in one direction and half sliding in the other. No changes were observed in the I-band. Locker and Leet (1975) explained this result by proposing that the gap filaments secured the thick filaments only to one end of the sarcomere in an alternating pattern. Upon excessive stretch, the gap filaments would bear most of the force, and the thick filaments would be dislocated from their regular position in the A-band.

Locker and Leet (1976) theorized that each gap filament forms a core to a thick filament, emerging at only one end of the thick filament and running through the Z-line to terminate as a core to a second thick filament in the adjacent sarcomere. Thus, gap filaments would form the elastic component in muscle, providing continuity of structure through the Z-line, but not through the sarcomere. Locker (1982) has since proposed that gap filaments determine the tensile strength of the myofibril in the raw or cooked state and are therefore important in meat tenderness.

Since the identification of gap filaments, considerable effort has been made to isolate an elastic protein(s) which

could be the primary components of the gap filaments. Maruyama (1976) isolated a highly insoluble intracellular elastic protein and named it connectin. Furthermore, Maruyama (1976) and Maruyama et al. (1977) proposed that this elastic protein was responsible for structural continuity and tension transmission in skeletal muscles. Maruyama et al. (1977) reported that connectin comprised about 5% of the total myofibrillar proteins in rabbit psoas muscle and it contained about 5% lipids and 1% sugars.

Maruyama et al. (1977) observed that when the SDS-soluble portion of connectin was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), a large portion of connectin did not penetrate into a 10% gel, indicating that it was an extremely large protein. Maruyama et al. (1980) slowly electrophoresed connectin on 2.5% SDS-gels and reported a mobility similar to that of a tetramer of myosin heavy chains, suggesting a molecular weight of approximately 800,000 daltons. More recently, Maruyama et al. (1981) proposed that two forms of connectin, alpha- and beta-connectin exist. Kimura and Maruyama (1983) reported that the molecular weights of connectin were 2.8×10^6 and 2.1×10^6 for the alpha and beta forms, respectively.

Maruyama et al. (1980) used immunofluorescence to localize connectin in myofibrils. Staining occured strongly

in the A-band regions and on the Z-line, and weakly in the I-band. It was concluded that connectin was located throughout the sarcomere. Based on that evidence, Maruyama et al. (1980) proposed a model which showed that connectin formed three-dimensional elastic nets that surrounded the entire sarcomere between the Z-lines. However, in a more recent model, Maruyama (1985) proposed that connectin existed as filaments spun from the Z-line, or proteins associated with the Z-line, that eventually branch out as nets or webs surrounding the ends of the thick filaments.

Wang and coworkers (Wang et al., 1979; Wang and Williamson, 1980) identified three large myofibrillar proteins and initially named them band 1, 2, and 3 in decreasing order of molecular weight as resolved by SDS-PAGE. Wang et al. (1979) collectively called the first two bands, titin and individually named the first band, titin-1 (T_1, $M_r = 1.4 \times 10^6$), and the second band, titin-2 (T_2, $M_r = 1.2 \times 10^6$). Wang and Williamson (1980) named band 3, which had a molecular weight of 5×10^5, nebulin. Together, these proteins make up approximately 15% of the total myofibrillar proteins.

Maruyama et al. (1981) confirmed on the basis of using SDS-PAGE, amino acid analysis, and indirect immunofluorescence techniques, that the high molecular weight components of connectin were identical to T_1 and T_2

as discovered by Wang et al. (1979). Purified titin has been prepared from myofibrils by Wang (1982) using gel permeation chromatography in the presence of SDS. Wang et al. (1984) and Trinick et al. (1984) have reported purification procedures for native T_2 by taking advantage of the protein's solubility in high NaCl and high pH solutions.

Titin and nebulin have been located in the myofibril with monospecific antibodies (Wang et al., 1979; Wang and Williamson, 1980). Wang et al. (1979) observed that titin antibodies labeled a wide zone at the A-/I-band junction, and also at the Z-line, M-line, and possibly throughout the entire A-band. Wang et al. (1979) concluded that transverse structures (M-line and Z-line) contained titin, that the titin containing structure was attached to the ends of the thick filaments, and that titin had variable affinity to antibody labeling depending on sarcomere length. LaSalle et al. (1983) examined titin localization with polyclonal antibodies to bovine skeletal muscle titin in isolated skeletal myofibrils. Immunofluorescence labeling showed intense labeling in the region of the A-/I-band junction with some labeling extending into the A-band.

Nebulin has been identified by indirect immunofluorescent localization studies as a major protein component of the myofibrillar N_2-line of skeletal muscle (Wang and Williamson, 1980; Ridpath et al., 1982). The

localization of the N_2-line as a transverse structure in the I-band, where the thin filament array changed from a square lattice at the Z-line to a hexagonal pattern near the A-/I-band junction, suggested that the N_2-line may regulate thin filament geometry (Franzini-Armstrong, 1970; Wang and Williamson, 1980). Wang and Williamson (1980) proposed that nebulin and the N_2-lines were attached directly or indirectly to elastic titin filaments within the myofibril.

Wang et al. (1984) and Wang and Williamson (1980) have suggested that titin and nebulin are the components of a third set of filaments in the sarcomere, since the properties of these proteins are not explained by the properties of the thick and thin filaments. The proposed third set of filaments are continuous, connecting Z-line to Z-line and are elastic along their entire length, except where they interact with inelastic structures such as the thick filament. Recently, Wang (1984) proposed a model which included titin and nebulin as constituents of a third filament lattice in the sarcomere. In that model, titin was associated with the thick filament, and nebulin was associated with titin and the thin filament forming an elastic connection between the A- and I-bands. Wang (1984) postulated that the elastic titin-nebulin lattice may function to provide continuity and support for the thick and thin filaments. In addition, this titin-nebulin lattice may

also have a role in the assembly and turnover of the thick and thin filaments. In fact, Wang et al. (1984) reported that titin is ideally suited as a component of an elastic lattice that serves as an organizing scaffold or template for thick and thin filaments.

Zeece et al. (1986) reported that titin and nebulin are highly susceptible to proteolytic degradation, especially by CAF. Lusby et al. (1983) showed that both titin and nebulin exist in beef muscle and these proteins are degraded during postmortem storage. Paterson and Parrish (1986) demonstrated with SDS-PAGE that the titin and nebulin in tender beef muscles, as determined by higher sensory panel tenderness scores and lower Warner-Bratzler Shear values, were more highly degraded than titin and nebulin in tough beef muscles. This indicates that titin and nebulin may have an important role in meat tenderness and perhaps, and important role in the water holding capacity of meat.

Effects of salt and phosphates on water holding capacity

Water is the primary constituent in meat systems accounting for approximately 75% of the weight of lean meats. The study of ways in which water is bound in meats is of considerable scientific interest and economic

importance. The water holding capacity (WHC) of meat is of scientific importance because it has been found to be closely related to palatability, tenderness, color, and other characteristics of meat quality (Hamm, 1960). The WHC of muscle tissue is of economic importance because meat and meat products are sold by weight, and loss of water from these products will result in economic loss to the meat industry.

Hamm (1960) concluded that water in muscle is bound in three basic ways and described those as bound, immobilized, and free. Bound water is that directly associated with the muscle proteins. Polar, hydrophilic side chains and undissociated carboxyl and amido groups of peptide bonds are responsible for holding this water very tightly (Hamm, 1960). Water in this fraction represents 4-5% of the total water in muscle tissue and can be removed only under severe drying conditions. The remaining water in muscle tissue can be either free or immobilized. Free water is that portion of water that is held in meat by surface tension or weak capillary forces. It is easily removed by drying, drip loss, or other physical forces. Much of the free water in a meat system exists as immobilized water. It is immobilized by the network of protein filaments in the myofibril. Hamm (1970) suggested that there is a continuous transition between the water that is immobilized by the myofibril

protein network and the free water that can be removed by low pressures. The WHC of a meat system is directly dependent on the ability of muscle tissue to maintain a majority of its water in the immobilized state. Changes in the amount of immobilized water, and hence the WHC of muscle tissue, can be caused by factors such as pH, ionic strength, and rigor state that alter the charge and configuration of the myofibrillar proteins.

Effect of sodium chloride

It is well known that sodium chloride (NaCl) increases the swelling of meat tissue. This swelling has been found to be significantly correlated to increased WHC (Bendall, 1954; Wierbicki et al., 1957; Sherman, 1961). Hamm (1960) reported the influence on meat homogenates of chlorides of different metals and sodium salts of a number of strong acids at different ionic strengths in a wide pH range. He found that these salts caused a number of different effects and found large differences in their respective expressible moisture values. The results can be summarized as follows: a) above the isoelectric point of protein, the addition of neutral salts, such as NaCl, causes a hydration effect, b) below the isoelectric point of protein, the addition of neutral salts results in the dehydration of meat, c) various ions affect the expressible moisture of meat to different

degrees and the differences are due to protein-ion interaction, d) the influence of added ions to the meat homogenates, increased hydration (less expressible moisture) in lyotropic progression is found, i.e., F < Cl < Br < I < CNS, and Ca < Mg < K < Na < Li (in the alkaline range).

Hamm (1960, 1970) emphasized the importance of the strength of ion binding to the protein as an explanation for the different effects of neutral salts on the WHC of meat. In the alkaline pH range, the hydrating effect of the anion is more pronounced than the hydrating effect of the cation. The stronger an ion is bound to the protein the greater the repulsion and the greater the hydration. On the acid side of the isoelectric point (pI), there is less repulsion and hence no hydration.

The nature of the binding of salt ions to proteins is believed to be mainly electrostatic due to the binding of oppositely charged salt ions to positively or negatively charged ion groups of the protein (Hamm 1960, 1970). According to this explanation, on the acidic side of the pI of muscle the binding of anions to the positively charged protein reduces the electrostatic repulsion between these positively charged groups by decreasing the net charge of the proteins. The result of this reaction is a tightening of the protein structure and an increase in expressible moisture. The stronger the ion is bound, the stronger will

be the effect of dehydration. On the basic side of the pI, because the protein is negatively charged, the binding of anions has the opposite effect. The result is a widening of the muscle structure, and consequently an increase in the immobilized water along with swelling. The more tightly the anion is bound, the stronger will be the hydration effect of the anion.

Schut (1976) proposed another mechanism by which neutral salts such as NaCl increase the swelling and WHC of meat. The chloride anion, being larger, would be less hydrated than the sodium cation. This would enable the chloride ion to come into closer proximity to the protein due to a smaller radius and less steric interference. The anion would have a large screening effect on positively charged residues remaining on the protein at pH values higher that the pI of the system, resulting in a net increase of negative charges and a repulsion of the protein strands creating a larger available space for the immobilization of free water in the muscle tissue. The opposite effect, i.e., smaller available space, would be observed at pH values below the pI of muscle (Schut, 1976).

Effect of salting meat in the prerigor state

The inclusion of prerigor meat into processed meat formulations is known to improve yield and quality of the

final product. In many cases, however, for certain technical reasons meat can not be processed immediately after slaughter. Hamm (1977) noted that salting of prerigor meat preserves the high WHC although it accelerates the breakdown of ATP. This effect of arresting the postmortem decrease of the WHC was not shown at NaCl concentrations lower than 1.8% (Hamm, 1981).

The addition of salt at pH values higher than the pI of meat causes a strong increase of WHC and swelling of muscle which is related to a shift of the pI of the myofibrillar protein to lower values. The formation of actomyosin complex during rigor will hinder this swelling effect of NaCl. Thus, the effect of NaCl in increasing the water holding properties of cooked or raw muscle homogenates is diminished with progressive development of rigor mortis (Hamm, 1981). Therefore, in order to obtain this beneficial presalting effect, the salt has to penetrate the tissue before the ATP concentration has fallen to a level of 1 μmol/g at which the onset of rigor mortis occurs (Honikel et al., 1981). It is thus important that the salt should penetrate the prerigor meat as quickly as possible. Fast salting can be obtained by sprinkling the salt on cuts of hot-boned meat and passing them through a meat grinder. It should be realized that grinding of meat, as well as the lowering the tissue temperature below about 10°C, hastens

the turnover of ATP and, consequently, the onset of rigor mortis (Hamm, 1981). Therefore, it is important to salt the prerigor meat before or immediately after grinding and before cooling of the ground meat.

It has been demonstrated by rheological measurements (Hamm, 1975) that the water holding properties of prerigor salted beef does not decrease for several days postmortem. The onset of rigor mortis is prevented by blocking of the interaction between actin and myosin. Hamm (1981) explained that it is probably due to a strong repulsion between adjacent protein molecules caused by the initial combined effect of relatively high ATP, high pH and high ionic strength. The salting of prerigor muscle induces an increased electrostatic repulsion due to increased negative net charge caused by the Cl^- ions and a decreased electrostatic repulsion due to thickness of the electric double layer. These two counteracting forces are initiated by the addition of NaCl to prerigor muscle. But the force of the former is stronger and thus, increased electrostatic repulsion is likely to be induced in the salted prerigor muscle (Hamm, 1981).

Hamm and van Hoof (1974) induced increased WHC by the addition of ATP to comminuted postrigor beef, adjusted to pH 6.9 in the presence of NaCl. In this case the combined effect of high ATP, high pH and high ionic strength produced

a considerable increase of WHC which remained at its high level, although the ATP was quickly hydrolyzed.

Another explanation for the presalting effect could be an inhibition of the uptake of Ca^{2+} by troponin-C caused by addition of the salt ions prior to the onset of rigor. Ca^{2+} ions released from the sarcoplasmic reticulum postmortem are bound by troponin-C. This induces the interaction between actin and myosin. Ca^{2+} release accelerates the ATP breakdown by additional activation of myosin ATPase (Bendall, 1973). This enzyme in turn promotes the interaction of actin and myosin. Thus, blocking this interaction should also block rigor mortis.

Effect of alkaline phosphates

Phosphates serve many useful functions as additives in food applications. Among these are functions in ion exchange reactions, as buffers, in interactions with other polyelectrolytes, as sequestrants of unwanted metal ions and as microbiological inhibitors (Ellinger, 1972). Ellinger (1972) lists seven major functions of phosphates in meats. These are: a) color preservation, b) increasing tenderness, c) increasing binding, d) increasing moisture retention, e) flavor improvement, f) prevention of off-flavor development, and g) prevention of microbial spoilage. The following treatise will deal entirely with the ability of alkaline

phosphates to improve the WHC of meat systems.

Classification of inorganic phosphates

Inorganic phosphates can be classified by the number of phosphorus atoms in the molecule. The classifications of phosphates important for use in meat products are orthophosphates, pyrophosphates, and straight-chained polyphosphates (Anonymous, 1982). The orthophosphates are the salts of orthophosphoric acid, and contain only one phosphorus atom. Only the sodium and potassium salts of these phosphates are approved for use in meat products. Pyrophosphate or diphosphate molecules are the salts of pyrophosphoric acid and are made up of two esterified phosphate molecules. This type of phosphate structure is referred to as a "condensed" structure. The pyrophosphates approved for use in meat products are sodium acid pyrophosphate, an acidic phosphate, and tetrasodium or tetrapotassium pyrophosphate, which are alkaline phosphates.

Phosphates consisting of three or more esterfied phosphorus atoms are referred to as polyphosphates, which may be in either straight-chained form or cyclic form. The straight-chain phosphates are the most commonly used in food applications. Sodium and potassium tripolyphosphates contain three phosphorus atoms and the sodium salt of this phosphate is the most commonly used in conventional

processed meat products. Sodium hexametaphosphate is a long, straight-chained phosphate that has been incorrectly named, since the designation "metaphosphate" is only correct for cyclic molecules. The name "hexametaphosphate" has been officially replaced by the USDA with the name "sodium polyphosphate glassy" (Grahm's salt) and "sodium metaphosphate, insoluble" (Maddrell's salt). Food grade hexametaphosphate has an average of 10-15 phosphorus atoms and is frequently used in blends with tripolyphosphate and other phosphates (Ellinger, 1977; Everson, 1985).

Hamm (1960, 1970) summarized the effects of alkaline phosphates on the increase in WHC in meat products as: a) an increase in pH, b) an increase in ionic strength, c) the ability to chelate divalent metal ions, d) the ability to bind to meat proteins, and e) the ability to dissociate actomyosin.

Effect of pH

WHC of a meat system is at a minimum when the pH of the system is near the pI of actomyosin. The pI is the pH at which the net charge of the protein is zero, which results in the formation of a maximum number of salt bridges between protein chains. For a meat system, this is in the pH range of 5.0-5.4. Increasing or decreasing the pH away from the pI will cause an increased in WHC by creating a charge

imbalance. An abundance of either positive or negative charges will result in a repulsion of similarly charged groups on proteins, and an increase in the ability to retain moisture (Hamm, 1960).

Several authors (Hamm, 1960; Hellendoorn, 1962; Mahon, 1961; Sherman, 1961; Wierbicki et al., 1962) have concluded that of the important effects of alkaline phosphates on meat is the elevation of the pH of meat away from the pI, thus increasing the WHC of meat. Ellinger (1972) reported that 1% solutions made from phosphates approved for use in meat products would result in the following pH values:

	pH
sodium acid pyrophosphate	4.2
sodium polyphosphate, glassy	7.0
sodium or potassium tripolyphosphate	9.8
tetrasodium or tetrapotassium pyrophosphate	10.2

However, due to the buffering capacity of meat, the effect of phosphates on the pH of meat is much less pronounced. In fact, Mahon (1961) reported that the addition of 0.2-0.5% tripolyphosphate increased the pH of meat by only 0.1-0.3 units. Puolanne and Matikkala (1980) showed that relatively small changes in pH (such as those reported by Mahon, 1961) in meat products containing salt and various types of phosphates had a pronounced positive effect on meat WHC.

Shults et al. (1972) working on the effects of various phosphates at 0.5% concentration in beef semimembranosus muscle, found that pyrophosphate was the most effective phosphate in raising both the pH of the muscle and its WHC. Sodium tripolyphosphate was next most effective in raising meat pH and WHC whereas hexametaphosphate was found to have a very small effect on meat pH and WHC.

Effect of ionic strength

Inorganic phosphates ionize in water to form polyelectrolytes. This ionization of polyphosphates in solutions increases the overall ionic strength of the meat system, which has been shown to result in an increase in meat tissue WHC (Hellendoorn, 1962). However, the increase in ionic strength produced by phosphates is difficult to measure due to the fact that they are not completely dissociated in solution (Van Wazer, 1970). Recently, Trout and Schmidt (1983) reported on the degree of dissociation of 6 different phosphates, commonly used in meat products, whose chain lengths varied from 1 to 22. They stated that with increasing phosphate chain length there was a concurrent reduction in the degree of dissociation of the phosphate molecule. When the ionic strength of a 0.5% phosphate solution was computed, taking the degree of dissociation into consideration, there was a reduction in

ionic strength (from 0.2 to 0.9) with increasing phosphate chain length. The lower ionic strength of the longer chain length phosphates may explain the results of Shults et al. (1972). They found that hexametaphosphate, with a chain length range of 12-14, was less effective than pyrophosphate and tripolyphosphate in increasing meat WHC.

The increase in WHC produced by increases in ionic strength has been found to occur over a relatively narrow ionic strength range. Mahon (1961), Hellendoorn (1962), Ishiorishi et al. (1979), and Trout and Schmidt (1983; 1984; 1986a) have shown that the increase in WHC (both with and without phosphate) begins when the total ionic strength is 0.4 and continues until the ionic strength is 0.6. Phosphates allowed in meats are not able to increase ionic strength to this degree, but are effective when used in conjunction with ~2% NaCl. This is in agreement with the results of Gillett et al. (1978) and Puolanne and Ruusunen (1980).

Chelation of divalent cations

The ability of alkaline phosphates to chelate metal ions, particularly calcium and magnesium ions, was postulated by Hamm (1960) as being an important factor in increasing the WHC of meat. He theorized that magnesium and calcium ions bind to proteins, causing tightening of the

molecular network structure and release of water. According to Schut (1976), it is quite certain that phosphates react with alkali earth metals from muscle tissue. This reaction was demonstrated by the extraction of Ca^{2+} from muscle by phosphate solutions. Differences have been reported, however, for the extractability of Ca^{2+} and Mg^{2+} by phosphate solutions. Some disagreement exists as to whether or not Mg^{2+} would be extracted by phosphate solutions. Baldwin and deMan (1968) found that added phosphate removes Ca^{2+} but not Mg^{2+} from their bound state with beef muscle proteins. Hamm (1970) pointed out that even though Mg^{2+} is not extractable some reaction may occur. He suggested a reaction by which Mg^{2+} shared one of its valences with the protein and its other valence bound to the phosphate. Hamm (1970) postulated that by this reaction, the tightening effect of Mg^{2+} could be negated resulting in loosening of the structure of the protein and an increase in WHC.

Evidence against the importance of the metal ion chelation effect of phosphates is that other strong chelating agents, such as ethylene diamine tetracetate (EDTA) or oxalate do not increase the WHC of muscle tissue (Sherman, 1961). Hamm (1970) suggested that Sherman's (1961) observations could be explained if one assumed that the "wide-spreading" EDTA molecule could not approach protein-bound divalent cations because of stearic

interference. However, Inklaar (1967) also demonstrated that polyphosphates did not reduce the amount of calcium or magnesium actually bound to the meat proteins. It must be concluded that chelation of calcium and magnesium ions is not a major factor in the increase of WHC produced by alkaline phosphates.

Binding to meat proteins

The binding of phosphate anions to meat proteins, to either specific sites as occur in actomyosin or to nonspecific positively charged protein side groups, has been postulated as an explanation for the increase in meat WHC (Hamm, 1970). Phosphate binding results in an increase in the net negative charge of the protein, which in turn leads to greater protein-protein repulsion within the myofibril and consequently to increased WHC (Hamm, 1970). Naus et al. (1969) have shown that myosin bound two moles of pyrophosphate per mole of myosin, but actomyosin bound only one mole. From this they concluded that each myosin molecule had 2 phosphate binding sites, but that one site was made unavailable due to the binding of actin to myosin. One point which tends to reduce the possible role of this type of binding in meat WHC is that it is independent of ionic strength, in the range of 0.1 to 0.6. Bendall (1954), Hellendoorn (1962), and Trout and Schmidt (1984, 1986a) have

shown that as ionic strength is increased from 0.1 to 0.6 with phosphates, there is a concurrent increase in meat WHC.

The non-site specific binding of phosphates to meat proteins may be important in increasing meat WHC. A point in favor of this is that all types of phosphates bind to proteins, but to varying degrees depending on the phosphate type and the particular protein (Lyons and Siebenthal, 1966; Vandegrift and Evans, 1981). This type of binding may help explain why the different phosphate types are effective in increasing meat WHC, but to varying degrees.

Dissociation of actomyosin

The increase in WHC produced by the presence of pyrophosphate was suggested by Bendall (1954) as being due to the increased solubility of muscle proteins as a result of the pyrophosphate induced dissociation of actomyosin. A prerequisite for this dissociation is the presence of low levels of magnesium ions. These Mg^{2+} ions bind to the myosin molecule and in so doing allow the pyrophosphate ions to bind to myosin. Calcium, however, is not believed to be involved in the binding of pyrophosphate to myosin (Kiely and Martonosi, 1968). Once pyrophosphate is bound, it dissociates actomyosin into actin and myosin with a subsequent release of orthophosphate (Granicher and Portzehl, 1964).

This ability to dissociate actomyosin is a property of pyrophosphate and not tripolyphosphate (Hamm, 1970). Similarly, Fukazawa et al. (1961) reported pyrophosphate to have a "specific effect" on extraction of proteins with 0.6 M NaCl Weber-Edsall solution. The addition of pyrophosphate caused considerably greater extraction of proteins than tripolyphosphate or hexametaphosphate from native or denatured myofibrils and at three different pH's (5.6, 6.4, and 7.0). Grabowski and Hamm (1979) also indicated that pyrophosphate increased the solubility of myofibrillar proteins. Regenstein and Stamm (1979) reported the extraction of proteins from chicken muscle with pyrophosphate to be greater than would be expected from its ionic strength contribution. The results of this study may have been due to the effect of pyrophosphate on the dissociation of the actomyosin complex.

Fukazawa et al. (1961) and Yasui et al. (1964a,b) also demonstrated that the pyrophosphate anion was the most effective anion causing dissociation of actomyosin and that the longer chain phosphates could only exert similar activity if they had been hydrolyzed to pyrophosphate either by enzymatic activity or by the pH of the solution. These investigators reported that pyrophosphate immediately promoted the dissociation of actomyosin while the tripolyphosphate required more time to act. This suggested

to Yasui et al. (1964b) that the tripolyphosphate had to first be hydrolyzed to pyrophosphate before the effect was noticed. Hexametaphosphate took even longer, again suggesting the necessity for hexametaphosphate to be hydrolyzed to pyrophosphate. Sutton (1973), in a study of the hydrolysis of phosphates, has shown by phase contrast photomicrographs, gross conformational changes in cod muscle with the addition of tripolyphosphate. It was suggested that this effect was due to tetrasodium pyrophosphate, resulting form the rapid hydrolysis of tripolyphosphate in muscle.

Although this appears to be a possible explanation for the effect of different phosphates in meat products, it does have a shortcoming. If the effectiveness of tripolyphosphate is due to it being hydrolyzed to pyrophosphate, then the effectiveness of tripolyphosphate is dependent on the time dependent hydrolysis. However, Ranken (1976), Poulanne and Ruusunen (1980), and Trout and Schmidt (1983) have reported that increasing the amount of time from preparation to thermal processing had no effect of the WHC of meat products containing tripolyphosphate.

Hydrolysis of phosphates in meat

Awad (1968) reported that tripolyphosphate was hydrolyzed at a faster rate than pyrophosphate in beef

muscle. He suggested that this was due to the fact that the tripolyphosphate structure resembles ATP more closely than does pyrophosphate, and that the enzyme system of muscle which hydrolyzes ATP may find tripolyphosphate a more similar substrate with which to interact. Neraal and Hamm (1977a,b) studied the occurrence of tripoly- and pyrophosphatases in beef sternomandibularis muscle and found the pyrophosphatase activity to be much lower that the tripolyphosphatase activity. Sutton (1973) also reported greater tripolyphosphatase activity than pyrophosphatase activity in beef sternomandibularis muscle.

Hamm and Neraal (1977c) showed that the addition of sodium chloride affected the phosphatase activity of beef muscle. Sodium chloride activated the tripolyphosphatase activity of minced postrigor beef muscle and may be optimally effective at a level of 4-5%. In contrast, Hamm and Nerall (1977a) found the pyrophosphatase activity to be reduced with increased concentration of added sodium chloride. Hamm and Neraal (1977b) reported that sodium chloride increased the maximum velocity (V_{max}) for tripolyphosphatase and decreased the V_{max} of pyrophosphatase.

Synergism with sodium chloride

The combination of sodium chloride and alkaline phosphates is reported to have a synergistic effect on the WHC of meat tissue (Hamm, 1970; Ranken, 1976; Trout and Schmidt, 1983). Sherman (1961) reported that the combination of salt and phosphate caused greater absorption of sodium and chloride than would be expected from the contributions of salt and phosphate to ion absorption when added alone. Mahon (1961) explained this by showing that the addition of 4-5% NaCl gave proteins a net negative charge. This was due to the net negative charge of proteins on the alkaline side of the pI of meat, which resulted in the preferential absorption of sodium (Na^+) ions. The net positive charge would cause the protein to more strongly attract the negatively charged phosphate anions.

Offer and Trinick (1983), Knight and Parsons (1984), Voyle et al. (1984), and Lewis et al. (1986) studied, with light and electron microscopy, the synergistic effects of NaCl and alkaline phosphates on the structure of muscle tissue. Offer and Trinick (1983) observed, with phase contrast microscopy, that isolated rabbit myofibrils swell very substantially in NaCl solutions similar to those used in meat processing, and concluded that the myofibrils were the major sites of water uptake in meat. They further reported that in the absence of pyrophosphate a

concentration of 0.8M NaCl was required for maximal swelling. Under these conditions, there was partial extraction of myosin from the center of the A-band. In the presence of pyrophosphate, a much lower concentration of NaCl (0.4M) was required for maximal swelling and extraction of the A-band was nearly complete. Removal of the A-band progressed from the ends towards the center. Offer and Trinick (1983) suggested that the synergistic effect of pyrophosphate was due to its ability to dissociate the actomyosin complex more completely that NaCl alone. They concluded that while the major constraint to myofibril swelling was the actomyosin complex, maximal myofibril swelling may depend on the integrity of the Z-line and M-line regions of myofibrils.

Knight and Parsons (1984) found similar synergistic effects on beef myofibrils isolated from beef sternomandibularis muscle, when pyrophosphate was combined with NaCl. However, they also indicated that isolated beef myofibrils behaved variably and attributed that to the presence of different muscle fiber types in the sternomandibularis muscle. Voyle et al. (1984) soaked pork longissimus muscle in NaCl solutions, with and without pyrophosphate, and reported that in the presence of both NaCl and pyrophosphate, pork tissue WHC was higher then when NaCl alone was present. Also, electron microscopy revealed

that pork tissue incubated in NaCl plus pyrophosphate, underwent complete or nearly complete extraction of the A-bands from myofibrils at the surface of the incubated meat. In the presence of NaCl alone, no extraction of the A-band occured. Voyle et al. (1984) attributed those structural changes to the ability of pyrophosphate to dissociate actomyosin and solubilize myofibrillar proteins.

Lewis et al. (1986) also studied the effects of NaCl and various alkaline phosphates on muscle tissue and in general agreement with Offer and Trinick (1983) and Voyle et al. (1984), they reported that the presence of phosphates produced increased WHC above that of NaCl alone. Unlike others however, pyrophosphate was not more effective than tripolyphosphate in increasing WHC. Also, in contrast to the conclusions of Trout and Schmidt (1983, 1984, 1986a), Lewis et al. (1986) found that the contribution of phosphates to the ionic strength of the meat system was not important in improving meat WHC. In addition, they believed that the dissociation of actomyosin and the sequestering action of phosphates were not important factors in explaining the behavior of the phosphates in that study. Rather, Lewis et al. (1986) concluded that the ability of phosphates to increase pH seemed to be the most important characteristic in increasing muscle tissue WHC.

PART I.

RESPONSE OF BEEF *STERNOMANDIBULARIS* MUSCLE TO VARIOUS SALT AND PYROPHOSPHATE TREATMENTS

ABSTRACT

The effects of sodium chloride (NaCl) and pyrophosphate (PP) were examined by treating beef tissue and isolated myofibrils with various concentrations of NaCl, with and without 10mM PP. Isolated beef myofibrils swelled to as much as 155% of their original diameters in the presence of 1.0M NaCl. With the addition of 10mM PP, the NaCl concentration necessary to achieve maximal swelling was reduced to 0.7M NaCl. Gel electrophoresis showed that higher NaCl concentrations (1.0M > 0.7M > 0.4M) increased the extraction of titin, myosin and other myofibrillar proteins from beef tissue and that the inclusion of 10mM PP to NaCl solutions enhanced the extraction of those proteins. Beef tissue water-holding capacity (WHC) was increased by higher NaCl concentrations and the presence of 10mM PP. Increased myofibrillar protein extraction was associated with increased beef myofibril swelling and increased beef muscle WHC.

INTRODUCTION

Fresh meat contains about 75% water, and the retention of this, and added water, during storage and further processing is of great importance to the meat industry. First, water retention is essential for the meat product palatability attributes of juiciness and tenderness. Second, water retention is economically important because losses of water result in a decreased amount of marketable product. This retention of moisture in meat and meat products is termed water-holding capacity (WHC), and the importance of WHC to meat quality has been extensively reviewed by Hamm (1970) and Ranken (1976).

The possible mechanisms by which sodium chloride (NaCl) and alkaline phosphates, such as pyrophosphate, improve the WHC of meat have been extensively reviewed (Hamm, 1960, 1970; Trout and Schmidt 1983). The addition of NaCl increases meat product WHC by increasing the ionic strength of the meat system (Hamm 1960). This enhances electrostatic repulsions, which cause a loosening of the myofibrillar structure so that there is more space for water to be trapped. Trout and Schmidt (1983) reported that alkaline phosphates can increase meat WHC by several methods. These include increasing meat system pH and/or ionic strength, chelating divalent metal ions, binding of phosphate anions to proteins or dissociation of the actin-myosin interaction.

Together, NaCl and alkaline phosphates are more effective in increasing WHC than either one alone inasmuch as they serve to disrupt muscle tissue, enabling meat to retain more moisture (Lewis, 1981).

Offer and Trinick (1983) studied the disruptive effect of NaCl and pyrophosphate on isolated rabbit psoas myofibrils and theorized that changes in the water content of meat originated from changes in the volume of myofibrils. They observed, with phase-contrast microscopy, that myofibril swelling occurred in the presence of NaCl solutions typical of those used in meat processing and that, if PP was added, swelling occurred at a lower NaCl concentration and that there was greater extraction of protein from the A-band. Offer and Trinick (1983) theorized that the observed changes in myofibril volume might be due to the disruption of structural constraints, such as Z-lines, M-lines and actin-myosin crossbridges, that exist within the myofibril.

Wang et al. (1979) and Wang and Williamson (1980) discovered two extremely large myofibrillar/cytoskeletal proteins and named them titin and nebulin, respectively. Wang (1984) theorized that, together, titin and nebulin may form a cytoskeletal network that holds the thick and thin filaments in an elastic framework within the myofibril. Offer and Trinick (1983) did not study the effects of NaCl

and PP on titin and nebulin nor did they consider titin and nebulin as possible physical constraints involved in regulating changes in myofibril volume.

The objectives of this study were: (1) to examine, by use of phase-contrast microscopy, the extent of beef myofibril swelling in the presence of various concentrations of NaCl or NaCl+10mM PP; (2) to detect, with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), changes in beef myofibrillar/cytoskeletal proteins, such as titin and nebulin, caused by treatment with NaCl or NaCl+10mM PP; and (3) to relate myofibril swelling and myofibrillar/cytoskeletal protein changes to a physical measure of beef muscle WHC.

MATERIALS AND METHODS

Preparation of myofibrils

Beef myofibrils were prepared by using a modification of the procedure of Knight and Parsons (1984). Beef sternomandibularis (STM) muscle was obtained immediately postmortem, vacuum packaged and held at 10°C for 24 h. Myofibrils were prepared by homogenizing 4 g of chopped STM for 30 s in 40 ml of chilled (4°C) buffer containing 0.1M KCl, 2mM $MgCl_2$, 1mM EGTA, 0.5mM dithiothreitol, 10mM K phosphate, pH 7.0. Myofibrils were isolated by centrifugation at 2000 x g for 5 min. Homogenization, followed by centrifugation, was repeated three times. Myofibrils were stored in homogenizing buffer at 4°C until phase-contrast microscopy was completed.

Irrigation of myofibrils

Myofibrils were irrigated as described by Offer and Trinick (1983). A drop of isolated myofibrils was applied to a microscope slide and gently covered with a coverslip. Treatment solutions were drawn between the slide and coverslip from a pool on one side of the coverslip by a piece of filter paper touching the opposite side. Myofibrils were irrigated with a series of increasing NaCl

concentrations, 0.1M, 0.4M, 0.7M and 1.0M NaCl, with or without 10mM tetra sodium pyrophosphate (PP), in 1mM $MgCl_2$, 10mM Na acetate, pH 5.5. Salt solutions were stored at 4°C, and the myofibril irrigation took place at 20°C. Myofibrils were observed with a Zeiss photomicroscope with a 100x phase-contrast objective and recorded photographically at a magnification of 400x. Myofibrils were photographed before irrigation (zero seconds = 0 s) and after 3 min. of irrigation (180 seconds = 180 s). Myofibril diameters were measured with an eyepiece micrometer. Percentage myofibril swelling was calculated as: (final diameter/initial diameter) x 100 = % swelling.

Preparation of SDS-PAGE protein fractions

Eight grams of ground STM was placed in 50 ml centrifuge tubes, and 24 ml from one of the NaCl or NaCl+10mM PP solutions was added. These muscle/NaCl/NaCl+PP homogenates were stored at 4°C for 24 h. Three protein fractions were prepared from these homogenates for SDS-PAGE analysis. The first fraction for SDS-PAGE analysis was prepared from an aliquot of the muscle/NaCl/NaCl+10mM PP homogenate (muscle/NaCl/NaCl+10mM PP fraction = MSF). Purified myofibrillar proteins were prepared (Goll et al., 1974) by homogenizing the 8 g of STM from the muscle/NaCl/NaCl+10mM PP homogenate in a Waring blender and

centrifuging at 1500 x g for 10 s with five changes of standard salt solution (SSS) (100mM KCl, 20mM K phosphate, pH 6.8, 2mM $MgCl_2$, 2mM EGTA, 1mM NaN_3) and two changes of SSS plus 1% Triton-X 100. The supernatant from the first SSS wash in this myofibrillar protein preparation was retained as the second fraction (supernatant fraction = SNF) for SDS-PAGE analysis. Protein content of the MSF, SNF and final myofibril suspensions were determined according to the biuret procedure of Robson et al. (1968). After biuret analysis, the final myofibril suspensions were centrifuged at 1500 x g for 10 min, and the supernatants were decanted and replaced with an equal volume of SDS-sample buffer (2% w/v SDS, 10mM Na phosphate, pH 7.0). Myofibrils were resuspended in the SDS-sample buffer and homogenized by hand using a Kontes glass tissue homogenizer. The homogenates were placed in a water bath at 50°C for 20 min, then centrifuged at 1500 x g to remove any undissolved material. The supernatants from these final myofibrillar protein suspensions, which contained purified myofibrillar proteins, were retained as the third fraction (myofibrillar protein fraction = MFF) for SDS-PAGE analysis.

SDS-PAGE

MSF, SNF and MFF fractions were heated for 20 min at 50°C in tracking dye (7.5% v/v 2-mercaptoethanol, 1.5% w/v

SDS, 15% sucrose, 0.03% bromophenol blue, 15mM 2-N-morpholinoethanesulfonic acid, pH 6.5). SDS-PAGE was performed according to the method of Paterson and Parrish (1986) using 3.2% polyacrylamide (acrylamide/bisacrylamide, 37:1) slab gels (pH 8.0) and 10% polyacrylamide (acrylamide/bisacrylamide, 37:1) slab gels (pH 8.9) with the dimensions of 16cm x 18cm x .15cm, at 30mA per gel. The 10% slab gels were stained overnight in an excess of 0.2% w/v Coomassie brilliant blue R-250, 7% v/v glacial acetic acid and 40% v/v methanol, then destained in an excess of the same solution excluding the Coomassie brillant blue; 3.2% slab gels were silver stained by using the procedure of Heukeshoven and Dernick (1985). Gels were stained by fixing in a 30% v/v ethanol, 10% v/v glacial acetic acid solution for 3 h. Fixation was followed by two washes in a 10% ethanol solution and three washes in water. Staining was done in a 0.1% $AgNO_3$ solution for 1 h. After the gels were rinsed with water for 20 s, development was initiated with a 2.5% w/v Na_2CO_3 solution containing 0.2% v/v formaldehyde. The development reaction was stopped with a 1% v/v glacial acetic acid solution.

Water-holding capacity

Eight grams of ground STM was placed in 50 ml centrifuge tubes, and 24 ml from one of the NaCl or

NaCl+10mM PP solutions was added. These muscle/NaCl/NaCl+PP homogenates were stored at 4°C for 24 h. Centrifuge tubes were then centrifuged at 1000 x g for 5 min. The supernatants were decanted, and the centrifuge tubes were reweighed and placed in an 80°C water bath for 20 min. After cooking, tubes were cooled to room temperature and centrifuged at 1000 x g for 5 min. The supernatants were decanted, and the centrifuge tubes were reweighed to determine percentage cooking yield. The formula for determining percentage cooking yield is:

$$\% \text{ cook yield} = \frac{\text{tube weight after cooking and decanting}}{\text{tube weight before cooking}} \times 100$$

RESULTS

Effect of NaCl or NaCl+10mM PP on beef myofibril swelling

When beef myofibrils stored in homogenizing buffer (pH 7.0) were irrigated with 0.1M NaCl, pH 5.5, for 180 s, myofibril diameter shrank slightly (Table 1). The addition of 10mM PP increased myofibril swelling, but the increase was not significant. Also, the increased myofibril swelling observed after increasing the NaCl concentration from 0.1M to 0.4M NaCl was not statistically significant. However, a significant ($P<0.05$) increase in myofibril swelling occurred when myofibrils were irrigated with 0.4M NaCl+10mM PP as compared with those irrigated with 0.4M NaCl alone. Indeed, myofibril swelling, in the presence of 0.4M NaCl+10mM PP was not different from swelling observed of myofibrils irrigated with solutions containing 0.7M NaCl alone. Consistent with myofibril swelling data observed with 0.4M NaCl, myofibrils irrigated with 0.7M NaCl+10mM PP exhibited significant ($P<0.05$) increases in swelling greater than those irrigated with 0.7M NaCl alone. In addition, myofibril swelling measured in the presence of 0.7M NaCl+10mM PP was not increased when myofibrils were irrigated with 1.0M NaCl solutions, with or without 10mM PP (Table 1). Thus, maximal beef myofibril swelling was achieved by irrigation with 1.0M

NaCl or with 0.7M NaCl+10mM PP. The addition of 10mM PP did not affect maximal myofibril swelling, but it did lower the NaCl concentration necessary to achieve it from 1.0M to 0.7M NaCl.

Effect of NaCl or NaCl+10mM PP on myofibril structure

Figure 1 displays the effects of irrigating myofibrils with increasing NaCl concentrations. It is evident that no detectable structural changes have occurred in beef myofibrils after irrigation with 0.1M or 0.4M NaCl solutions. In addition to the swelling that had taken place, major structural changes began to occur in the presence of 0.7M NaCl. These changes appeared to be located primarily in the region of the A-band. Indeed, after 180 s of irrigation in 0.7M NaCl, much of the center of the A-band had been extracted. However, the A-/I-band junction appeared to be resistant to extraction by the NaCl solution. Also, a narrow band of protein, detected in the M-line region of the A-band, was somewhat resistant to extraction. Increasing the NaCl concentration to 1.0M caused further protein extraction from the A-band center, including the previously resistant M-line region. But the A-/I-band region was resistant to extraction by the 1.0M NaCl solution. Z-lines remained intact after irrigation with all concentrations of NaCl, but the Z-lines did become more

diffuse after beef myofibrils were irrigated with 0.7M and 1.0M NaCl solutions.

Figure 2 shows beef myofibril structural changes after irrigation with NaCl solutions containing 10mM PP. Like myofibrils irrigated with 0.1M NaCl, those irrigated with 0.1M NaCl+10mM PP showed no variation from the regular myofibril structure. However, unlike myofibrils irrigated with 0.4M NaCl, myofibrils irrigated with 0.4M NaCl+10mM PP did exhibit structural changes. These changes occurred primarily in the A-band region and consisted of the extraction of protein from the ends of the A-bands rather than from the A-band centers as was observed in myofibrils irrigated with NaCl alone. A more pronounced protein extraction from the ends of A-bands was exhibited by myofibrils irrigated with 0.7M NaCl+10mM PP. A-bands from myofibrils irrigated with 1.0M NaCl+10mM PP solutions were almost totally extracted. Only a narrow protein band in the M-line region remained at the center of the A-bands. The results from Figures 1 and 2 clearly show that the irrigating solutions containing just NaCl preferentially extracted myofibrillar protein from centers of myofibril A-bands; whereas, irrigating solutions containing both NaCl and 10mM PP extracted myofibrillar proteins from the ends of A-bands.

SDS-PAGE

SDS-PAGE could not be performed on irrigated beef myofibrils to detect protein changes, so STM was incubated in NaCl or NaCl+10mM PP solutions to create similar conditions to those found during myofibril irrigation. Three protein fractions were evaluated, and Figures 3, 4 and 5 are the 3.2% SDS-gel electrophoretograms, showing changes in the myofibrillar proteins with molecular weights greater than myosin heavy chain (MHC). Figures 6, 7 and 8 are the 10% SDS-gel electrophoretograms, showing the changes in the myofibrillar proteins with molecular weights less than MHC.

Figure 3 is a 3.2% SDS-gel electrophoretogram of the MSF protein fraction, showing the proteins extracted from beef tissue incubated in NaCl or NaCl+10mM PP solutions. Titin is a very large myofibrillar protein that constitutes about 10% of the total myofibrillar protein (Wang et al., 1979). Wang et al. (1984) reported that titin migrated on SDS-gels as a closely spaced doublet, the members of which were, T_1 (M_r, $1.4x10^6$) and T_2 (M_r, $1.2x10^{6)}$. In Figure 3, as NaCl concentrations increased from 0.1M to 1.0M NaCl, more of the titin doublet was extracted from the beef STM. That was evidenced by the increased intensity of the titin protein bands observed at 0.4M, 0.7M and 1.0M NaCl, respectively. The addition of 10mM PP to NaCl solutions

helped to more effectively extract titin than solutions with NaCl alone. The resultant titin protein bands were clearly more intense from NaCl+10mM PP treatments than were the titin bands from treatments containing only NaCl.

Nebulin, another recently discovered myofibrillar protein, constitutes about 5% of all myofibrillar protein and has a molecular weight of $5x10^5$. Like the titin extraction, it seemed that nebulin was more effectively removed with higher NaCl concentrations; however, unlike titin, NaCl solutions extracted more nebulin than NaCl+10mM PP solutions (Figure 1). There were other protein bands located below nebulin that exhibited greater band intensity at the higher NaCl concentrations and particularly so when NaCl and 10mM PP were combined. It is not known if these protein bands are sarcoplasmic or myofibrillar proteins or if they are degradation products of titin or nebulin.

Figure 4 is the 3.2% SDS-gel electrophoretogram of the SNF protein fraction. These are the proteins that were not directly extracted from the beef STM homogenate but, rather, were released from the tissue after one blender homogenization step. The SNF fraction responded like the MSF fraction inasmuch as more titin was present on SDS-gels as the NaCl concentration increased. Also, the titin protein bands were more pronounced when 10mM PP was combined with NaCl than when NaCl was used alone. The small amount

of nebulin detected by the 3.2% SDS-gels of the SNF fraction appeared more prominently at the higher NaCl concentrations in the absence of 10mM PP.

The 3.2% SDS-gel electrophoretogram in Figure 5 showed the MFF protein fraction that remained in the beef STM after removal of the proteins in the MSF and SNF fractions. The decreased titin band intensity at the higher NaCl concentrations, especially when 10mM PP was combined with NaCl, indicated that the greater amounts of titin detected in the MSF and SNF fractions were actually due to the extraction effects of NaCl or NaCl+10mM PP rather than to the presence of more protein in those samples. Nebulin protein bands also showed decreased intensity at the higher NaCl concentrations but only when 10mM PP was absent.

Figures 6 and 7 are the 10% SDS-gel electrophoretograms of the MSF and SNF protein fractions, respectively. Those gels contained both sarcoplasmic and myofibrillar proteins, which made the results more difficult to interpret. Nonetheless, the major myofibrillar protein change detected by the 10% SDS-gels was the increased amount of MHC at the higher NaCl concentrations. Additionally, for both the MSF and SNF fractions, more MHC was detected when NaCl and 10mM PP were combined than when NaCl was used alone. The greater presence of MHC at the higher NaCl concentrations for both NaCl and NaCl+10mM PP treatments was expected because both

treatments removed a significant portion of beef myofibril A-bands as the NaCl concentration increased (Figures 1, 2). Also, myofibrillar proteins such as M-line protein and myosin light chain-1 (MLC-1) were detected in greater amounts on 10% SDS-gels at the higher NaCl concentrations for both the MSF and SNF protein fractions. The 10% SDS-gel electrophoretogram of the MFF protein fraction in Figure 8 showed the decreased amounts of detectable MHC, M-line protein and MLC-1 as a result of the increased amounts of those proteins in the MSF and SNF protein fractions. Treatment with NaCl or NaCl+10mM PP did not affect the appearance of the myofibrillar proteins α-actinin or actin on the SDS-gels (Figures 6, 7 and 8). However, the SDS-gel in Figure 8 showed that, at higher NaCl concentrations, with and without 10mM PP, the band intensity of the myofibrillar proteins troponin-T, tropomyosin and troponin-I decreased. Thus, these myofibrillar proteins were extracted by NaCl or NaCl+10mM PP from beef STM along with titin, nebulin, MHC, M-line protein and MLC-1.

Effect of NaCl or NaCl+10mM PP on WHC

Means for percentage cooked yields, used as a measure of WHC for beef STM incubated with various NaCl or NaCl+10mM PP solutions, are presented in Table 2. Percentage cooked yields for beef STM increased significantly ($P<0.05$) as NaCl

concentration increased from 0.1M through 1.0M NaCl. In addition, the presence of 10mM PP combined with NaCl significantly ($P<0.05$) improved cooked yields at each NaCl concentration. Indeed, 10mM PP improved cooked yields to the extent that the cooked yield for 0.1M NaCl+10mM PP was not different from that of 0.4M NaCl, and the cooked yield of 0.4M NaCl+10mM PP was not different from that of 0.7M NaCl. Also, the improvement in percentage cooked yields for beef STM caused by the presence of 10mM PP was more pronounced at the NaCl concentrations of 0.7M and 1.0M NaCl because both 0.7M NaCl+10mM PP and 1.0M NaCl+10mM PP produced significantly ($P<0.05$) higher cooked yields than STM treated with 1.0M NaCl alone.

DISCUSSION

Myofibril swelling and structural changes

Offer and Trinick (1983) reported that rabbit psoas myofibrils changed little in diameter or structure when irrigated with 0.1M through 0.5M NaCl solutions. However, at 0.6M NaCl, myofibrils swelled considerably, and the centers of A-bands were partly extracted. Maximal myofibril swelling was observed at 0.8M NaCl, and at 1.0M NaCl, the centers of myofibril A-bands were almost totally extracted. Conversely, when Offer and Trinick (1983) irrigated myofibrils with NaCl solutions containing 10mM PP, swelling and structural changes occurred at lower NaCl concentrations. Maximal myofibril swelling was lowered from 0.8M NaCl to 0.4M NaCl in the presence of 10mM PP. Also, structural changes were first observed at 0.4M NaCl+10mM PP rather than at 0.6M NaCl when PP was absent. They reported that, in the presence of 10mM PP, the ends of rabbit myofibril A-bands were always extracted first rather than the A-band centers when PP was absent. Thus, in the present study, the decreased NaCl concentration required for maximal myofibril swelling when 10mM PP was present and the differences in A-band protein extraction due to the presence or absence of 10mM PP closely agree with the results reported by Offer and Trinick (1983).

Knight and Parsons (1984) reported similar swelling and structural changes after irrigating beef myofibrils in various NaCl concentrations, with or without 10mM PP. Their results indicated that the presence of 10mM PP decreased the NaCl concentration required for maximal swelling and that NaCl solutions preferentially extracted protein from A-band centers, whereas NaCl+10mM PP extracted protein from the ends of A-bands. Voyle et al. (1984) soaked pork muscle in solutions containing 0.6M NaCl, with or without 10mM PP. Electron microscopy of the pork incubated in 0.6M NaCl+10mM PP, pH 5.5, revealed that A-bands were partly extracted from its ends, but this occurred only near the surface of the incubated tissue. No extraction of the A-band occurred in pork muscle incubated in 0.6M NaCl without PP.

The structural changes in myofibril A-bands reported by previous researchers, and as observed in the present study, may be explained by the effects of NaCl and PP on the interaction between myosin and actin. High NaCl concentrations will depolymerize myosin and extract it from the A-band (Trinick and Cooper, 1980); however, NaCl will not cause full dissociation of actomyosin (Hamm, 1970). This may explain why high NaCl concentrations extracted the centers of A-bands and why the A-/I-band regions were relatively resistant to extraction. Bendall (1954) reported that PP, in the presence of Mg^{2+}, was a weak dissociating

agent of the actomyosin complex. Hence, the combination of high NaCl concentrations (0.4M, 0.7M, and 1.0M NaCl) and 10mM PP resulted in the weakening and dissociating of the actomyosin interaction and, thus, the extraction of protein from the ends of the A-bands. Therefore, if the actomyosin interaction is a major physical hindrance to maximal myofibril swelling as suggested by Offer and Trinick (1983), then the synergistic effect that NaCl and PP have on the actomyosin interaction would explain why the presence of 10mM PP significantly lowered the NaCl concentration (from 1.0M NaCl to 0.7M NaCl) required for maximal myofibril swelling in the present study.

SDS-PAGE

Knight and Parsons (1984) and Offer and Trinick (1983) used phase-contrast microscopy to examine myofibrils irrigated with NaCl or NaCl+PP. But only Offer and Trinick (1983) used SDS-PAGE in conjunction with their microscopical observations on myofibrils. However, they examined only myofibrillar proteins with molecular weights of less than MHC, thus excluding the high-molecular-weight proteins titin and nebulin from their study. Recent work by Paxhia and Parrish (1987) indicated that titin and nebulin in pork tissue may be altered in the presence of NaCl and phosphate. That report agrees with the results of the present study

that may link these proteins, especially titin, to increased myofibril swelling and increased tissue WHC.

Titin and nebulin have been located in the sarcomere by using monospecific antibodies. Wang et al. (1979) reported that titin antibodies labeled at the A-/I-band junction and also at the M-line and Z-line. LaSalle et al. (1983) showed that titin antibodies labeled the gap regions between the A- and I-bands of highly stretched beef muscle. Wang and Williamson (1980) showed that nebulin antibodies labeled the myofibril at the location of the N_2-line, which is located in the I-band region between the Z-line and A-/I-band junction. On the basis of locations and properties of titin and nebulin, Wang (1984) theorized that titin was associated with the thick filament and that nebulin was associated with titin and the thin filament, forming an elastic connection between the A- and I-bands. This proposed interaction between titin and nebulin may provide a structural framework in the sarcomere, which, if altered, may affect myofibril swelling and WHC.

The phase-contrast micrographs and SDS-gel electrophoretograms in the present study support the research (Wang et al., 1979; LaSalle et al., 1983; Wang and Williamson, 1980) that indicated the location and function of titin and nebulin. In the presence of 10mM PP, NaCl solutions were shown to preferentially extract protein from

the ends of myofibrils where the A-/I-band junction is located. SDS-gels of the MSF and SNF fractions showed that more titin was released by NaCl+10mM PP solutions than by NaCl solutions, which extracted protein from the center of myofibril A-bands. Thus, if the primary location of titin is at the A-/I-band junction, then extraction of this area by NaCl+10mM PP would explain the increased intensity of titin bands on SDS-gels of beef tissue treated with NaCl+10mM PP. Conversely, because NaCl solutions extracted the A-band centers and not the A-/I-band junctions, less titin was evident on SDS-gels of the MSF and SNF fractions from beef tissue treated with NaCl solutions without 10mM PP.

Solutions containing NaCl+10mM PP, not only released more titin from beef muscle than the solutions with NaCl alone, but also caused maximal myofibril swelling at lower NaCl concentrations. This indicates that the removal (or partial removal) of titin from beef myofibrils may remove the structural restraint necessary for maximal myofibril swelling to occur. On the basis of results of the present study, the role that nebulin played in myofibril swelling was more difficult to determine and may have been less important than that of titin. SDS-gels of the MSF and SNF fractions showed that nebulin extraction was slightly improved at higher NaCl concentrations, but the SDS-gel

evidence was less consistent than that for titin. In addition, the myofibril structural changes that were observed with phase contrast microscopy took place primarily in the A-band rather than in the I-band region of the myofibril where nebulin is reportedly (Wang and Williamson, 1980) located.

Offer and Trinick (1983) used SDS-gels to show that high (0.6M to 1.0M NaCl) NaCl concentrations more effectively extracted C-protein, tropomyosin, troponin, α-actinin, actin and myosin from rabbit tissue than did low (<0.6M NaCl) NaCl solutions. In the presence of 10mM PP, those myofibrillar proteins were extracted at lower NaCl concentrations. Offer and Trinick (1983) concluded that the major difference between the effects of NaCl solutions, with and without PP, was that the former extracted myosin much more effectively. SDS-gels in the present study corraborate much of their results. That is, increased NaCl concentrations did more effectively extract MHC, M-line protein and MLC-1 from beef STM. Also, these proteins were more effectively extracted in the presence of NaCl+10mM PP.

Offer and Trinick (1983) placed much emphasis on the removal of physical restraints such as the actin-myosin interaction to achieve maximal myofibril swelling. Although the extraction of myosin may play a significant role in increased myofibril swelling, SDS-gels in the present study

indicated that the removal of titin from beef STM may be just as important. The extraction of titin by NaCl and NaCl+10mM PP may release the structural constraints imposed on myofibrils by titin to result in increased swelling of beef myofibrils observed in the present study.

Water-holding capacity

Trout and Schmidt (1986) concluded that increases in ionic strength and pH are the most important properties of phosphates in improving the WHC of meat. In the present study, the effect of pH was eliminated because all solutions had a pH of 5.5. Trout and Schmidt (1984) reported that the increase in WHC, with and without phosphate, began when the total ionic strength is 0.4 and continued until the ionic strength is 0.6. None of the alkaline phosphates can increase ionic strength to that extent, but they could when used in conjunction with NaCl levels (~2%) used in meat processing (Trout and Schmidt, 1984).

Ishioroshi et al. (1979) reported that increasing ionic strength with phosphates increased the solubility of proteins. In addition, Wang et al. (1984) have shown that titin and MHC were soluble in Guba-Straub solution (0.3M KCl, 0.15M K phosphate pH 6.7). The conditions in the present study may approximate those of the Guba-Straub solution, explaining the increased extraction of titin and

MHC from beef STM. That increased protein extraction, viewed on phase-contrast micrographs and shown on SDS-gels, caused by NaCl+10mM PP solutions was likely associated with the increased WHC observed in beef STM treated with the same NaCl+10mM PP solutions. This agrees with the work of Voyle et al. (1984), who used electron microscopy to show that 0.6M NaCl+10mM PP extracted more myofibrillar protein from pork tissue than 0.6M NaCl alone. They attributed that protein extraction with the increased WHC of the pork tissue treated with the NaCl+10mM PP solutions. It was evident that increased ionic strength and the dissociation of actomyosin, both caused by the addition of 10mM PP to NaCl solutions, generated increased solubilization and extraction of myofibrillar proteins that resulted in the increased WHC of the beef STM in the present study.

Conclusions

Offer and Trinick (1983) and Voyle et al. (1984) showed the association of increased myofibrillar protein extraction with increased myofibril swelling and subsequent increased WHC in meat. They also showed that the addition of 10mM PP to NaCl solutions improved protein extraction, myofibril swelling and WHC above NaCl alone. The present study confirmed these results by uniquely combining phase contrast microscopy, SDS-PAGE and WHC tests to show the effects of

NaCl and NaCl+10mM PP on beef muscle tissue. The results showed that higher NaCl concentrations increased beef myofibril swelling, increased myofibrillar protein extraction and improved tissue WHC. The addition of 10mM PP to NaCl solutions decreased the NaCl concentration required for maximal myofibril swelling. The presence of 10mM PP also increased protein extraction, especially of titin and MHC, and substantially improved tissue WHC. Most importantly, the present study showed that the extraction of titin seemed to be the most important event in regulating increased myofibril swelling and improved tissue WHC.

Table 1. Mean myofibril swelling values[a] of beef muscle irrigated with various NaCl and NaCl+10mM PP solutions[b]

Irrigating Solutions	% Myofibril Swelling (diameter)
0.1M NaCl	99.0[x]
0.1M NaCl+10mM PP	106.0[x]
0.4M NaCl	108.0[x]
0.4M NaCl+10mM PP	132.0[y]
0.7M NaCl	136.0[y]
0.7M NaCl+10mM PP	154.0[z]
1.0M NaCl	154.0[z]
1.0M NaCl+10mM PP	155.0[z]
S. E.	4.1

[a]n=20.

[b]100% = No increase in myofibril diameter.

[xyz]Means that bear unlike superscripts differ significantly ($P<0.05$).

Table 2. Mean percentage cooked yield values[a] of beef muscle treated with various NaCl and NaCl+10mM PP solutions[b]

Treatment Solutions	% Cooked Yields
0.1M NaCl	95.0^{u}
0.1M NaCl+10mM PP	105.0^{v}
0.4M NaCl	110.0^{v}
0.4M NaCl+10mM PP	128.0^{w}
0.7M NaCl	132.0^{w}
0.7M NaCl+10mM PP	157.0^{y}
1.0M NaCl	142.0^{x}
1.0M NaCl+10mM PP	171.0^{z}
S. E.	3.2

[a] $n=20$.

[b] 100% = Original tissue weight.

^{uvwxyz}Means that bear unlike superscripts differ significantly ($P<0.05$).

Fig. 1 - Phase-contrast micrographs of beef myofibrils after irrigation with various NaCl solutions.

Magnification = 2800x. 0 s = myofibril before irrigation; 180 s = myofibril after 180 s of irrigation

NaCl Irrigated Myofibrils

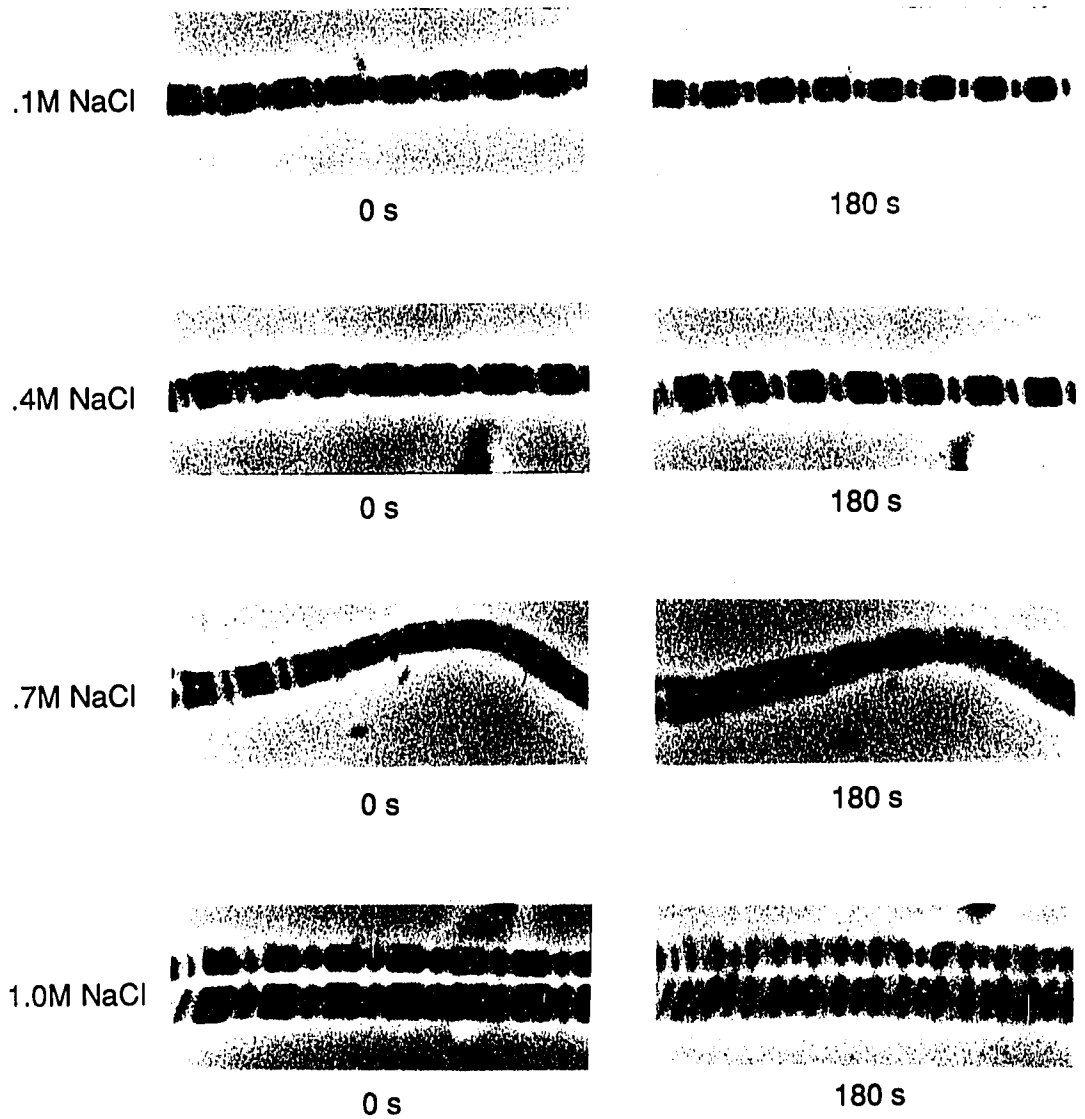


Fig. 2 - Phase-contrast micrographs of beef myofibrils after irrigation with various NaCl+10mM PP solutions.

Magnification = 2800x. 0 s = myofibril before irrigation; 180 s = myofibril after 180 s of irrigation

NaCl + 10mM Pyrophosphate Irrigated Myofibrils

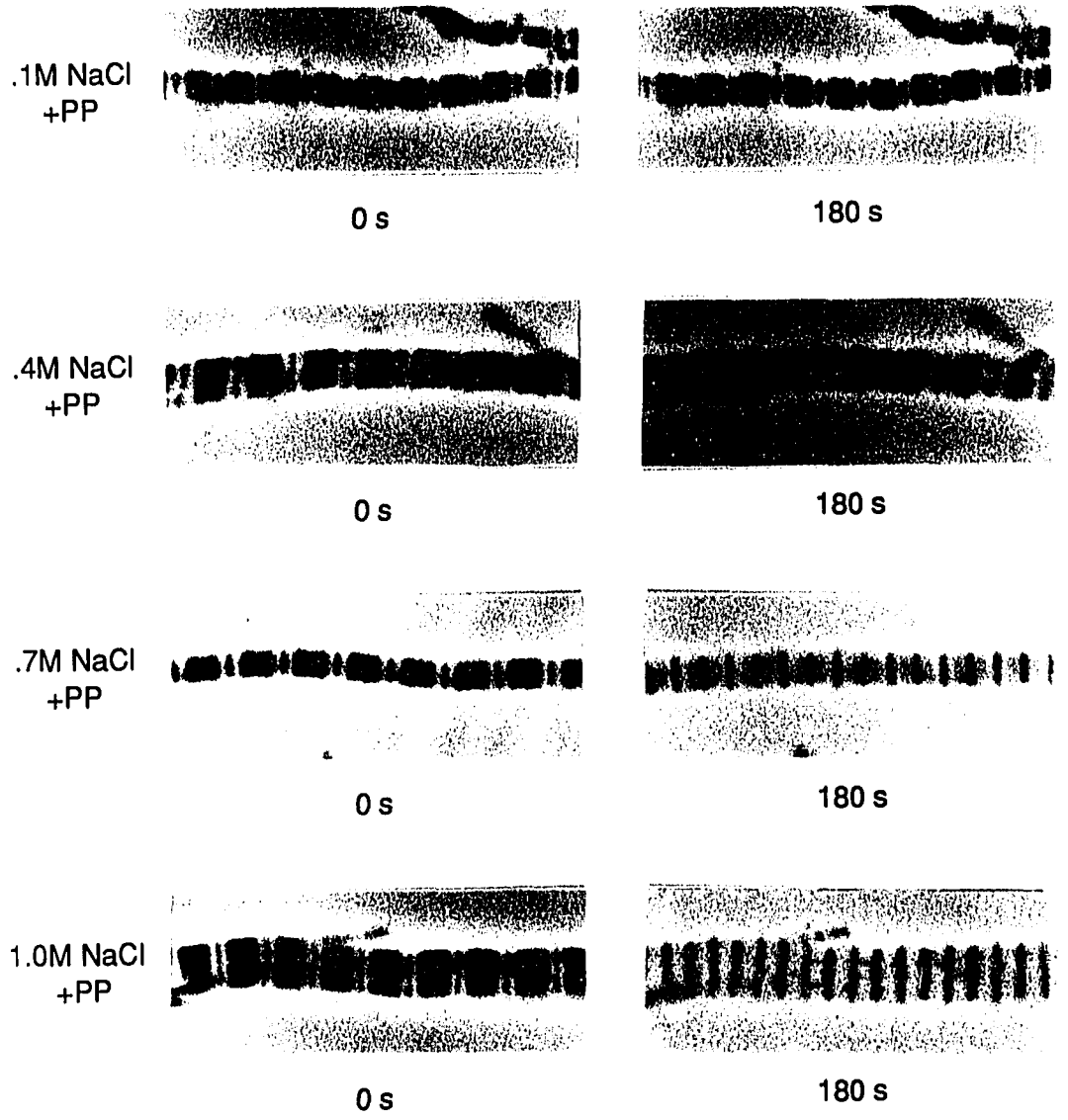


Fig. 3 - 3.2% SDS-gels (silver-stained) of beef muscle/NaCl/NaCl+10mM PP homogenates from beef sternomandibularis muscle after treatment with various NaCl and NaCl+10mM PP solutions

BEEF MUSCLE/NaCl/PP SOLUTION

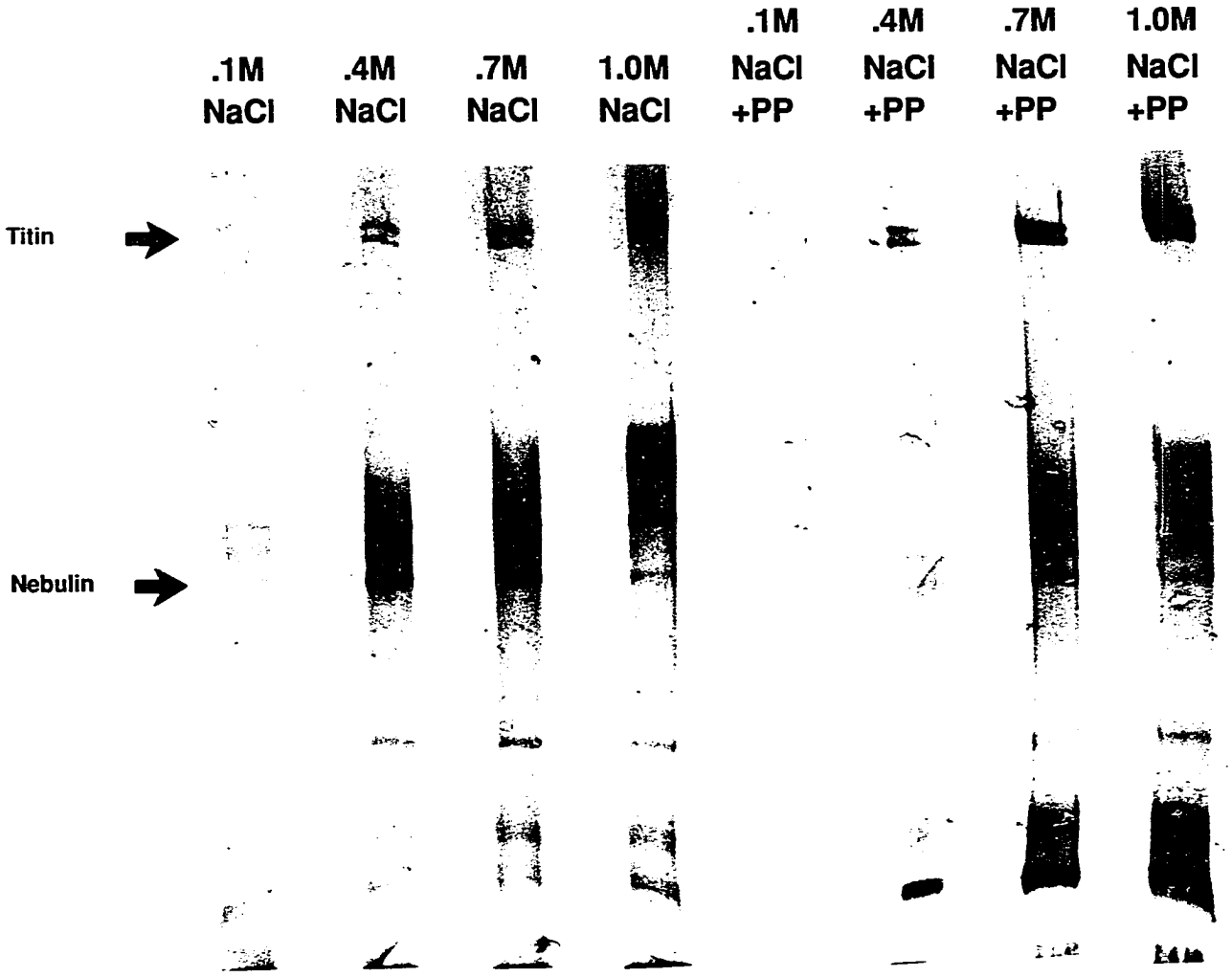


Fig. 4 - 3.2% SDS-gels (silver-stained) of supernatants from the myofibril protein purification procedure of beef sternomandibularis muscle after treatment with various NaCl and NaCl+10mM PP solutions

BEEF SUPERNATANT

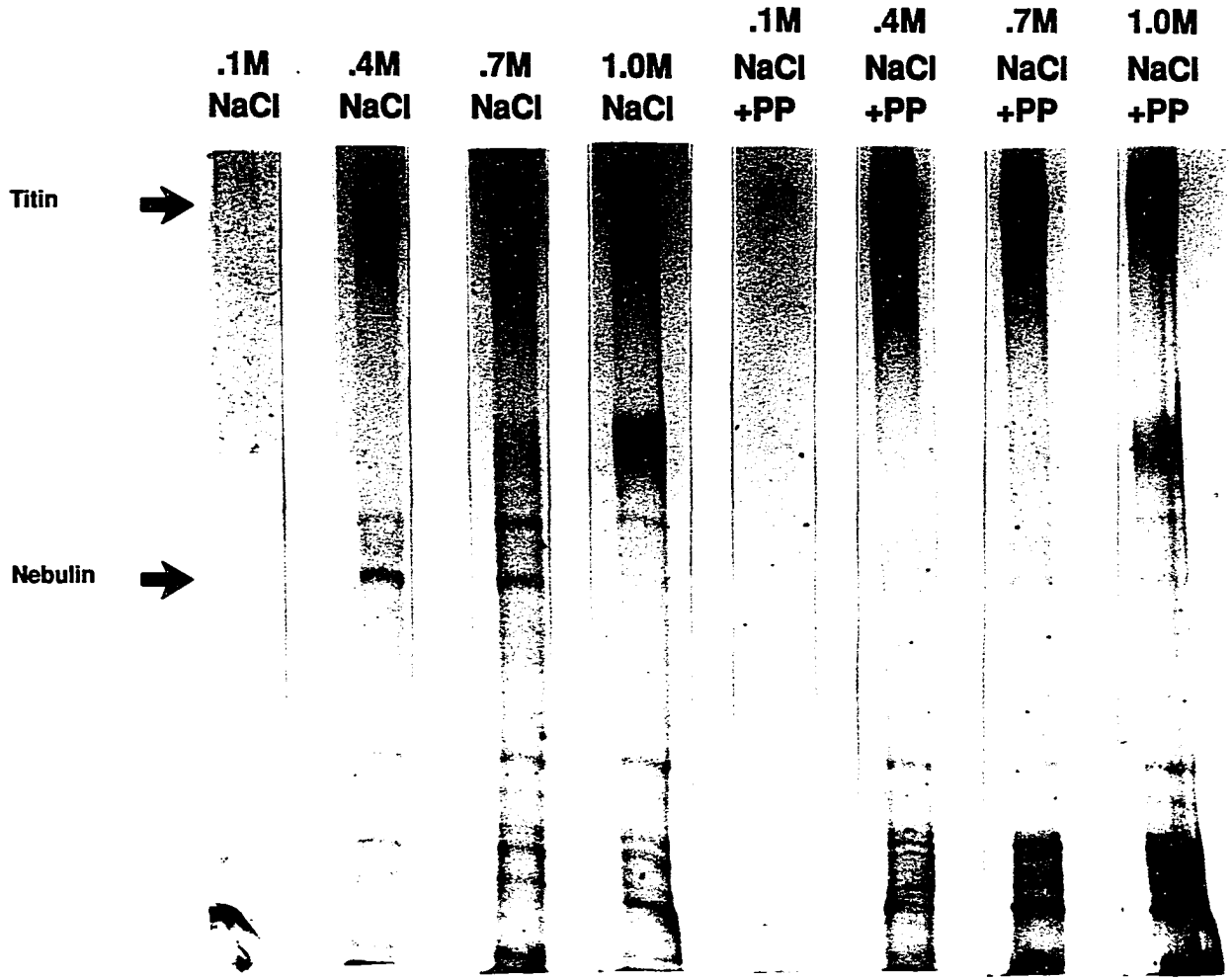


Fig. 5 - 3.2% SDS-gels (silver-stained) of purified myofibrillar/cyotskeletal proteins from beef sternomandibularis muscle after treatment with various NaCl and NaCl+10mM PP solutions

BEEF MYOFIBRILS

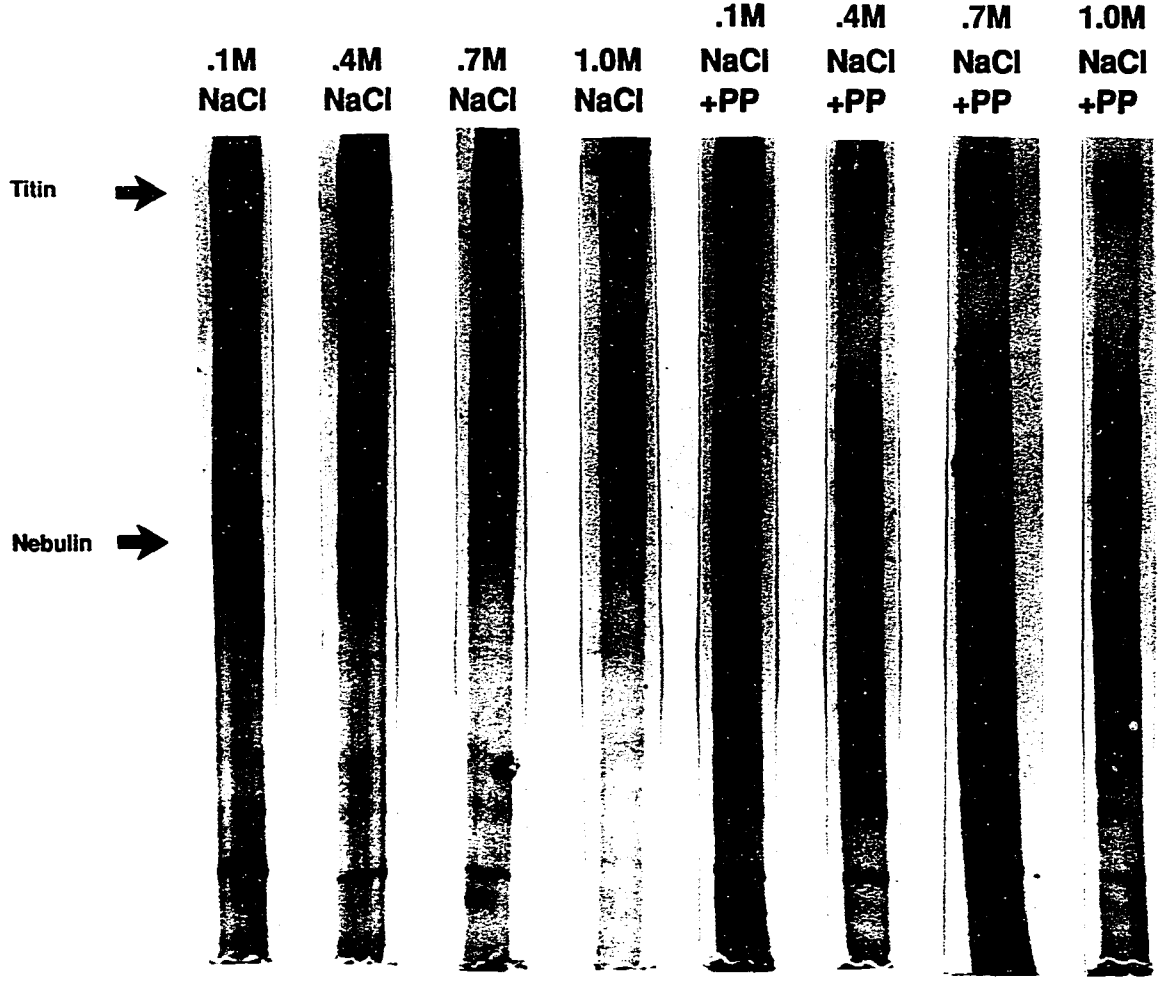


Fig. 6 - 10% SDS-gels (Coomassie blue-stained) of beef muscle/NaCl/NaCl+10mM PP homogenates from beef sternomandibularis muscle after treatment with various NaCl and NaCl+10mM PP solutions

BEEF MUSCLE/NaCl/PP SOLUTION

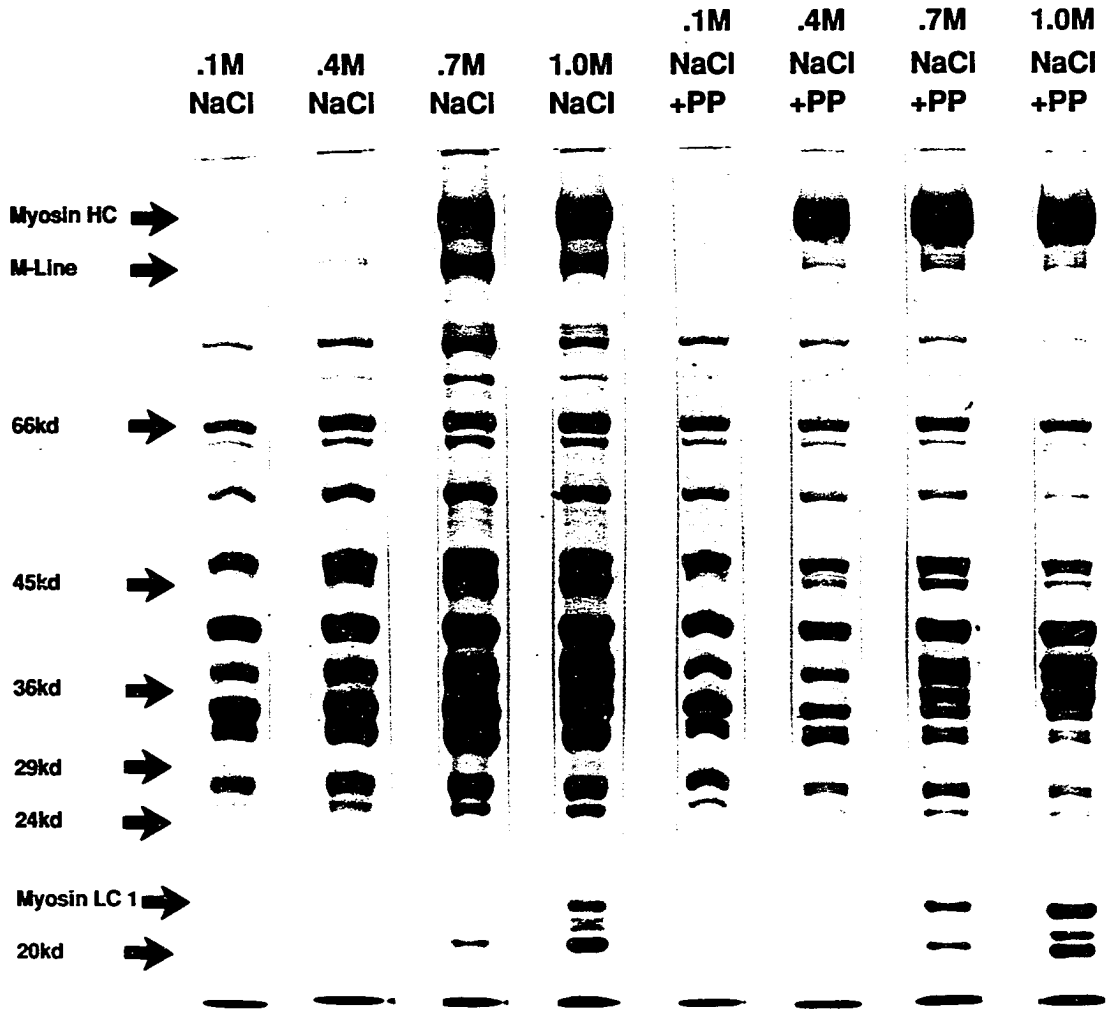


Fig. 7 - 10% SDS-gels (Coomassie blue-stained) of supernatants from the myofibril protein purification procedure of beef sternomandibularis muscle after treatment with various NaCl and NaCl+10mM PP solutions

BEEF SUPERNATANT

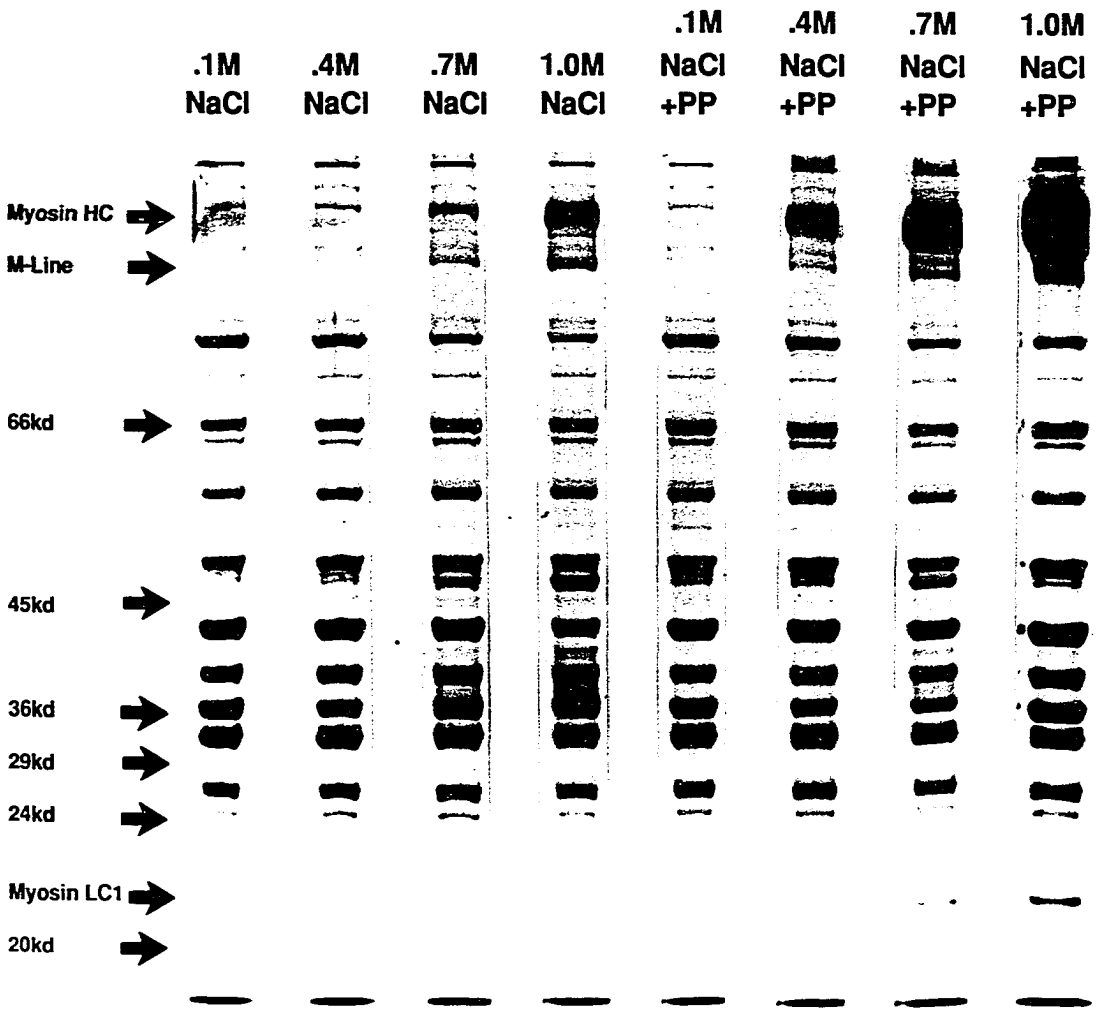
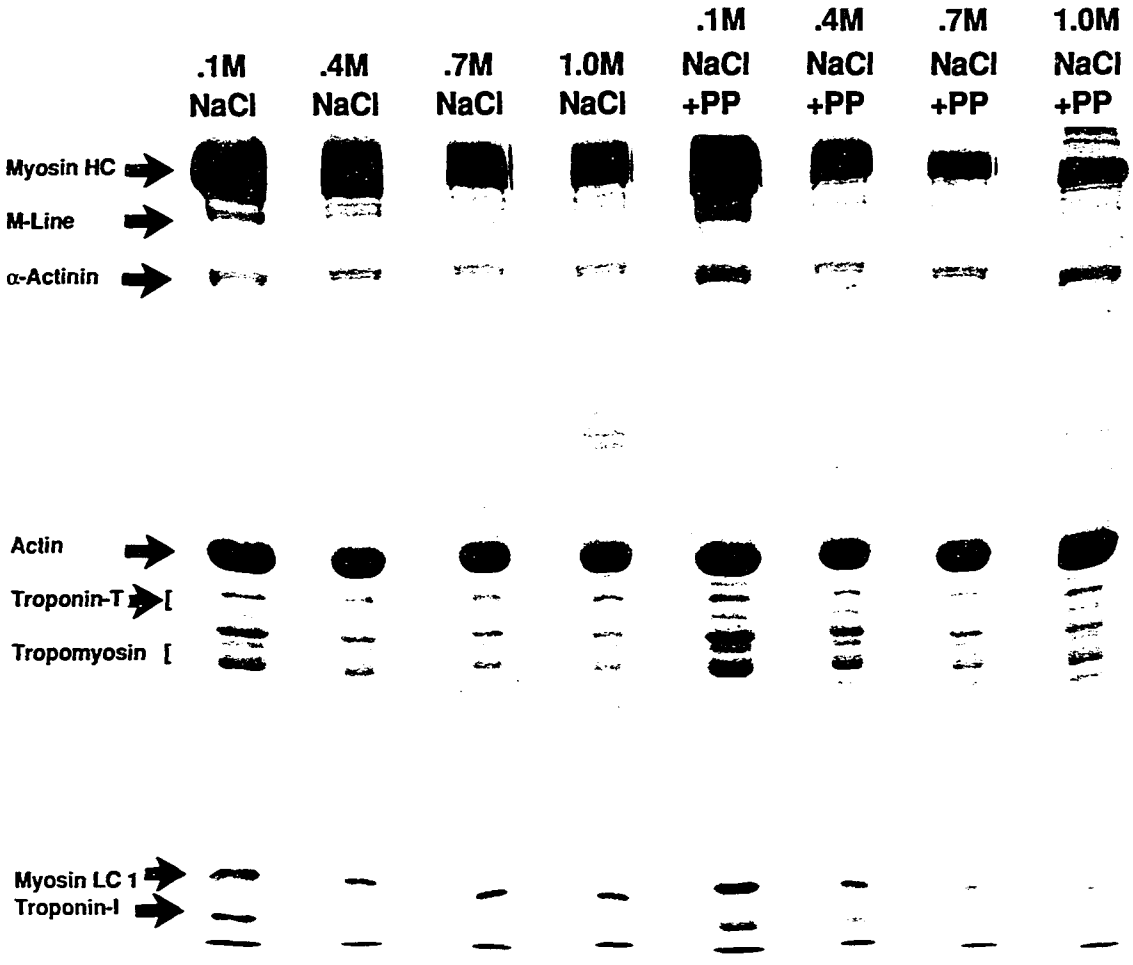


Fig. 8 - 10% SDS-gels (Coomassie blue-stained) of purified myofibrillar proteins from beef sternomandibularis muscle after treatment with various NaCl and NaCl+10mM PP solutions

BEEF MYOFIBRILS



REFERENCES

Bendall, J. R. 1954. The swelling effect of phosphates on lean meat. J. Sci. Food Agric. 5:468-475.

Goll, D. E., Young, R. B., and Stromer, M. H. 1974. Separation of subcellular organelles by differential and density gradient centrifugation. Proc. Recipr. Meat Conf. 27:250-259.

Hamm, R. 1960. Biochemistry of meat hydration. Adv. Food Res. 10:355-463.

Hamm, R. 1970. Interactions between phosphates and meat proteins. Pp. 65-82. In DeMann, J. M. and Melnychyn, P. (Eds). Symposium: Phosphates in Food Processing. AVI Publishing Co., Westport, CT.

Heukeshoven, J., and Dernick, R. 1985. Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. Electrophoresis 6:103-112.

Ishioroshi, M., Samejima, K. and Yasui, T. 1979. Heat-induced gelation of myosin: Factors of pH and salt concentrations. J. Food Sci. 44:1280-1284.

Knight, P. J., and Parsons, N. J. 1984. Variable response of beef myofibrils to salt solutions. Proc. Eur. Meet. Meat Res. Workers. 30:118-119.

LaSalle, F., Robson, R. M., Lusby, M. L., Parrish, F. C., Jr., Stromer, M. H., and Huiatt, T. W. 1983. Localization of titin in bovine skeletal muscle by immunofluorescence and immunoelectron microscope labeling. J. Cell Biol. 97:258a.

Lewis, D. F. 1981. The use of microscopy to explain the behavior of foodstuffs - a review of work carried out at the Leatherhead Food Research Association. Scanning Electron Microsc. III:391-404.

Offer, G., and Trinick, J. 1983. On the mechanism of water holding in meat: The swelling and shrinking of myofibrils. Meat Sci. 8:245-281.

Paterson, B. C., and Parrish, F. C., Jr. 1986. A sensory panel and sensory and chemical analysis of certain beef chuck muscles. J. Food Sci. 51:876-879.

Paxhia, J. M., and Parrish, F. C., Jr. 1987. Effect of various salt and phosphate treatments on the myofibrillar/cytoskeletal proteins from light and dark pork and poultry muscles. J. Food Sci. Submitted.

Ranken, M. D. 1976. The water holding capacity of meat and its control. Chem. Ind. 1976:1052-1057.

Robson, R. M., Goll, D. E., and Temple, M. J. 1968. Determination of protein in "tris" buffer by the biuret reaction. Anal. Biochem. 24:339-346.

Trinick, J., and Cooper, J. 1980. Sequential dissassembly of vertebrate muscle thick filaments. J. Mol. Biol. 141:315-321.

Trout, G. R., and Schmidt, G. R. 1983. Utilization of phosphates in meat products. Proc. Recipr, Meat Conf. 36:24-27.

Trout, G. R., and Schmidt, G. R. 1984. The effect of phosphate type, salt concentration and method of preparation on the binding in restructured beef rolls. J. Food Sci. 49:687-694.

Trout, G. R., and Schmidt, G. R. 1986. Effect of phosphates on the functional properties of restructured beef rolls: the role of pH, ionic strength, and phosphate type. J. Food Sci. 51:1416-1423.

Voyle, C. A., Jolley, P.D., and Offer, G. W. 1984. The effect of salt and pyrophosphate on the structure of meat. Food Microstruct. 3:113-126.

Wang, K. 1984. Sarcomere-associated cytoskeletal lattices in striated muscle, review and hypothesis. Pp. 315-369. In Shay, J. W. (Ed.). Cell and Muscle Motility. Vol. 2. Plenum Press, New York.

Wang, K., and Williamson, C. L. 1980. Identification of an N_2-line protein of striated muscle. Proc. Natl. Acad. Sci. USA 77:3254-3258.

Wang, K., McClure, J., and Tu, A. 1979. Titin: major myofibrillar components of striated muscle. Proc. Natl. Acad. Sci. USA 76:3698-3702.

Wang, K., Ramierez-Mitchell, R., and Palter, D. 1984. Titin is an extraordinarily long and flexible

myofibrillar protein. Proc. Natl. Acad. Sci. USA 81:3685-3689.

Journal Paper No. J-12727 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project 2711.

PART II.

EFFECTS OF VARIOUS SODIUM CHLORIDE AND ALKALINE PHOSPHATE TREATMENTS ON BEEF *STERNOMANDIBULARIS* MUSCLE

ABSTRACT

The effects of sodium chloride (NaCl), pyrophosphate (PP), tripolyphosphate (TPP) and sodium polyphosphate glassy (SPG) were examined by treating beef tissue and isolated myofibrils with various concentrations of NaCl, with and without 10mM PP, 10mM TPP or 10mM SPG. Beef myofibril swelling was greater when phosphates, especialy PP and TPP, were combined with NaCl than when NaCl alone was used. Gel electrophoresis showed that increasing NaCl concentrations resulted in the greater extraction of titin and myosin from beef tissue. More titin and myosin were extracted with NaCl+10mM PP than with NaCl+10mM TPP, NaCl+10mM SPG or NaCl alone. The water-holding capacity (WHC) of beef tissue increased as the NaCl concentration increased. However, the WHC of beef tissue was greater in the presence of a combination of 10mM phosphate+NaCl, particularly PP and TPP, than NaCl alone. Increased myofibrillar/cytoskeletal protein extraction, especially that of titin, was associated with increased beef myofibril swelling and increased beef tissue WHC.

INTRODUCTION

In the preceding paper of Paterson et al. (1987), the effects of various sodium chloride (NaCl) concentrations (0.1, 0.4, 0.7, and 1.0M NaCl) with or without 10mM pyrophosphate (PP) on the water-holding capacity (WHC) and myofibril swelling of, and myofibrillar protein extraction from, beef sternomandibularis muscle (STM) were investigated. The presence of 10mM PP in NaCl solutions decreased the NaCl concentration required for maximal myofibril swelling, increased WHC values and enhanced myofibrillar protein extraction, especially that of titin and myosin, from beef STM. The results from the preceeding study by Paterson et al. (1987) were in general agreement with those reported by Offer and Trinick (1983) and Voyle et al. (1984); however, none of these studies had evaluated the effects of alkaline phosphates other than pyrophosphate.

Phosphates are known to increase the functional properties of muscle tissue, especially WHC, but the extent to which this occurs varies due to the type of phosphates used (Shults et al., 1972; Trout and Schmidt, 1984). Trout and Schmidt (1984) reported as a generalization, the different phosphates increase functional properties of meat in the following order: pyrophosphate > tripolyphosphate (TPP) > sodium polyphosphate glassy (SPG). Lewis et al.

(1986) reported that muscle tissue containing PP or TPP had higher cooked yields than muscle containing other food grade phosphates such as SPG, tetrapolyphosphate and orthophosphate; however, the differences in cooked yields between phosphate types were not large. Changes in myofibril structure viewed with electron microscopy varied both within and between phosphate treatments. Unfortunately, Lewis et al. (1986) did not use gel electrophoresis to study differences in protein extraction due to the different phosphate treatments. Hence the objectives of this study were (1) to examine, with phase-contrast microscopy, the extent of beef myofibril swelling in the presence of various concentrations of NaCl or NaCl+10mM PP, NaCl+10mM TPP and NaCl+10mM SPG; (2) to detect, with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), changes in beef myofibrillar/cytoskeletal proteins, particularly titin, caused by the NaCl or NaCl+phosphate treatments; and (3) to relate myofibril swelling and SDS-PAGE-detected protein changes to beef <u>sternomandibularis</u> muscle WHC.

MATERIALS AND METHODS

Beef sternomandibularis (STM) was obtained 24 h postmortem from electrically stimulated, conventionally chilled (2°C) beef carcasses. Beef myofibrils were prepared for phase-contrast microscopy according to the method of Paterson et al. (1987). Isolated beef myofibrils were irrigated using the procedure of Paterson et al. (1987). Beef myofibrils were irrigated with a series of increasing NaCl concentrations: 0.30M, 0.47M, 0.64M and 0.80M NaCl. These NaCl solutions contained one of the following phosphate treatments: no phosphate, 10mM tetrasodium pyrophosphate, 10mM sodium tripolyphosphate or 10mM sodium polyphosphate glassy in 1mM $MgCl_2$, 10mM Na acetate, pH 5.5. Phase-contrast microscopy procedures, WHC tests and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample preparation procedures were the same as those used by Paterson et al. (1987). SDS-PAGE analysis was conducted according to the method of Paterson et al. (1987) with one exception. 12% polyacrylamide (acrylamide / bisacrylamide, 37:1) slab gels (pH 8.9) were used rather that the 10% polyacrylamide slab gels used by Paterson et al. (1987). The first protein fraction for SDS-PAGE analysis was prepared from an aliquot of the muscle/NaCl/PP/TPP/SPG homogenate (MSF). The supernatant

from the first homogenization step in the myofibrillar protein preparation was retained as the second protein fraction (SNF) for SDS-PAGE analysis. Purified myofibrillar proteins were retained as the third protein fraction (MFF) for SDS-PAGE analysis.

RESULTS

Beef myofibril swelling

Mean swelling values of isolated beef myofibrils, as measured by changes in myofibril diameter, are presented in Table 1. There was a significant ($P<0.05$) increase in myofibril swelling for each increase in NaCl concentration from 0.30M to 0.80M for the NaCl, NaCl+10mM PP, NaCl+10mM TPP and NaCl+10mM SPG irrigation treatments. The minimum myofibril swelling was recorded at the NaCl concentration of 0.30M for each of the four myofibril irrigation solution types, and the maximum myofibril swelling was recorded at 0.80M NaCl within each of the four irrigation solution types.

At the 0.30M NaCl concentration, the presence of 10mM phosphate with NaCl significantly ($P<0.05$) increased myofibril swelling above solutions containing just NaCl. However, the myofibril swelling values of the three 0.30M NaCl+10mM phosphate irrigating solution types did not differ from each other. The irrigating solutions containing 0.47M NaCl had significantly ($P<0.05$) lower myofibril swelling values than all of the 0.47M NaCl+10mM phosphate solutions. In addition, both the 0.47M NaCl+10mM PP and 0.47M NaCl+10mM TPP had significantly ($P<0.05$) higher swelling values than 0.47M NaCl+10mM SPG. That result repeated itself at the

0.64M NaCl level inasmuch as all of the 0.64M NaCl+10mM phosphate treatments produced higher ($P<0.05$) swelling values than 0.64M NaCl, and the myofibril swelling value of 0.64M NaCl+10mM SPG was significantly ($P<0.05$) lower than the swelling values of either 0.64M NaCl+10mM PP or 0.64M NaCl+10mM TPP. Maximal beef myofibril swelling for all treatment combinations was achieved by irrigation with 0.80M NaCl+10mM PP and 0.80M NaCl+10mM TPP both of which produced significantly ($P<0.05$) higher swelling values than either 0.80M NaCl+10mM SPG or 0.80M NaCl alone.

The effectiveness of adding 10mM PP or 10mM TPP to NaCl solutions can be shown in that the myofibril swelling values at 0.30M NaCl+10mM PP or TPP were equal to that of 0.47M NaCl. Myofibril swelling values for 0.47M NaCl+10mM PP or TPP were equal to that of 0.64M NaCl, and 0.64M NaCl+10mM PP or TPP were equal to those of 0.80M NaCl. The effect on myofibril swelling when 10mM SPG was added to NaCl solutions was less pronounced than 10mM PP or TPP, but 10mM SPG still produced increased myofibril swelling when compared to NaCl alone.

Effect of NaCl or NaCl+10mM phosphate on myofibril structure

Figure 1 shows the effects of various NaCl solutions on the structure of isolated beef myofibrils. There were no observed structural changes in beef myofibrils after three

minutes of irrigation with either 0.30M or 0.47M NaCl solutions. However, after irrigation with 0.64M NaCl, myofibril swelling, as well as protein extraction from the A-band region of the myofibrils, was observed. The protein was extracted primarily from the center of the myofibril A-band with the exception of the M-line region of the A-band which was resistant to extraction. In addition, the A-/I-band junctions were also resistant to extraction with 0.64M NaCl because the myofibril A-bands did not shorten from their ends. Increasing the NaCl concentration to 0.80M caused both increased swelling and protein extraction. Because the A-bands did not shorten from their ends, the A-/I-band junctions were apparently resistant to extraction by the 0.80M NaCl solution. The M-line region of the myofibril remained but to a lesser degree than in myofibrils treated with 0.64M NaCl. Z-lines remained intact after irrigation with all NaCl solutions, but the Z-lines did swell and become diffuse after irrigation with the 0.64M and 0.80M NaCl solutions.

Figure 2 shows beef myofibrils after irrigation with NaCl solutions containing 10mM PP. Myofibrils treated with 0.30M NaCl+10mM PP showed no structural changes; however, myofibrils irrigated with 0.47M NaCl+10mM PP did exhibit changes in myofibril appearance. These changes occurred in the A-bands of myofibrils and consisted of protein

extraction from ends of A-bands of myofibrils rather than from A-band centers as was observed in isolated myofibrils irrigated with NaCl alone. Furthermore, increased myofibrillar protein extraction and increased myofibril swelling were characteristic of beef myofibrils after irrigation with 0.64M NaCl+10mM PP and 0.80M NaCl+10mM PP. Myofibril A-bands were almost totally extracted leaving just a narrow protein band at the M-line located at the center of the myofibril A-bands. Figures 1 and 2 reveal that irrigating solutions containing just NaCl, extracted protein from the centers of myofibril A-bands while irrigating solutions containing NaCl+10mM PP extracted myofibrillar proteins from the ends of myofibril A-bands.

Figure 3 shows beef myofibrils that were irrigated with NaCl solutions containing 10mM TPP. The isolated beef myofibrils exhibited no changes in myofibril appearance after irrigation with 0.30M NaCl+10mM TPP but they began to show changes after irrigation with 0.47M NaCl+10mM TPP. The extraction of myofibrillar protein with this solution was more pronounced than in myofibrils irrigated with 0.47M NaCl alone. Actually, the myofibrils treated with 0.47M NaCl+10mM TPP resemble those treated with 0.64M NaCl because, although myofibrillar protein was extracted, the A-/I-band junctions and M-line regions of the myofibrils irrigated with 0.47M NaCl+10mM TPP remained resistant to

extraction. Myofibrils irrigated with 0.64M NaCl+10mM TPP had more protein extracted from their A-bands than did myofibrils irrigated with 0.47M NaCl+10mM TPP; however, A-/I-band junctions and the M-line regions were still resistant to extraction. Increasing the NaCl concentration in irrigating solutions to 0.80M, in the presence of 10mM TPP, did affect the A-/I-band junctions of irrigated myofibrils. Indeed, the myofibrils treated with 0.80M NaCl+10mM TPP underwent partial extraction of the A-/I-band junctions as evidenced by the shortening of A-bands from their ends.

The phase-contrast micrographs in Figure 4 showed that the irrigation of myofibrils with the 0.30M NaCl+10mM SPG solution did not affect the structural features of treated myofibrils. However, myofibrillar protein was extracted from the A-bands of myofibrils irrigated with 0.47M NaCl+10mM SPG and the extraction of protein increased as the NaCl concentration increased to 0.64M NaCl. Furthermore, the greatest amount of protein was extracted from SPG-treated myofibrils after irrigation with 0.80M NaCl+10mM SPG, but there was no visual evidence of A-band shortening like that observed in myofibrils treated with 0.80M NaCl+10mM TPP. Indeed, the A-/I-band junctions and M-line regions of myofibrils were resistant to extraction by 10mM SPG at all NaCl concentrations. The phase-contrast

micrographs in Figures 1-4 show that NaCl, by itself, was least effective in extracting myofibrillar protein and altering myofibril structure while NaCl+10mM PP was the most effective at protein extraction, expecially from the ends of myofibril A-bands. TPP extracted protein from the ends of myofibril A-bands (A-/I-band junctions) only at the 0.80M NaCl concentration, and 10mM SPG did not extract protein from myofibril A-/I-band junctions at any of the NaCl concentrations. Overall, myofibrils irrigated with NaCl+10mM TPP or NaCl+10mM SPG appeared more like myofibrils treated with irrigating solutions containing just NaCl than like myofibrils irrigated with NaCl+10mM PP.

SDS-PAGE

Three protein fractions from treated beef muscle were evaluated by SDS-PAGE and the results are shown in Figures 5-16, respectively. Figures 3 and 4 are the 3.2% SDS-gel electrophoretograms of the MSF protein fractions that show the high molecular weight proteins extracted from beef STM incubated in NaCl or NaCl+10mM phosphate solutions. Wang et al. (1984) reported that titin migrated on SDS-gels as a closely spaced doublet, the members of which were, T_1 (M_r, $1.4x10^6$) and T_2 (M_r, $1.2x10^6$). Wang and Williamson (1980) reported that nebulin had a molecular weight of $5x10^5$.

In Figure 5, more titin was extracted by the 0.64M and

0.80M NaCl solutions than by the 0.30M and 0.47M NaCl solutions; although, even at the higher NaCl concentrations, very little titin was actually extracted from beef muscle. The addition of 10mM PP to NaCl solutions greatly enhanced the extraction of the titin doublet from beef muscle. The resultant titin protein bands in Figure 5 from NaCl+10mM PP treatments were clearly more intense than were the titin bands from treatments containing only NaCl. In the presence of 10mM PP, higher concentrations of NaCl extracted more titin as evidenced by the more intense titin protein bands at 0.64M and 0.80M NaCl+10mM PP than at 0.30M and 0.47M NaCl+10mM PP.

Figure 6 shows, that for both NaCl+10mM TPP and NaCl+10mM SPG treatments, as the NaCl concentration increased from 0.30M to 0.80M, there was more titin doublet extracted from incubated beef muscle. Titin bands were more intense, at all NaCl concentrations, when 10mM TPP was present than when 10mM SPG was present in NaCl solutions. Thus, 10mM TPP was more effective in extracting titin than was 10mM SPG. Both NaCl+10mM TPP and NaCl+10mM SPG treatments exhibited greater titin extraction than the treatments containing NaCl alone. However, the 3.2% SDS-gel electrophoretograms in Figures 5 and 6 clearly show that NaCl+10mM PP was more effective at extracting titin from beef muscle at all NaCl concentrations than either NaCl+10mM

TPP or NaCl+10mM SPG.

Nebulin was more completely extracted as the NaCl concentration increased from 0.30M to 0.80M NaCl. Although the nebulin bands were slightly more intense in the NaCl+10mM PP treatments, all four solutions appeared to effectively remove nebulin from beef tissue and there were only marginal differences in nebulin extraction among the treatments when the intensity of nebulin bands was compared. There were other protein bands located below nebulin, in all four solution types, that exhibited greater protein band intensity at the higher NaCl concentrations. It is not known if these protein bands were individual sarcoplasmic or myofibrillar proteins or if they were degradation products of titin and nebulin.

Figures 7 and 8 are the 3.2% SDS-gel electrophoretograms of the SNF protein fraction. These were the proteins that were not directly extracted from beef STM homogenates but, rather, were released from the beef tissue after the first homogenization step when purified myofibrillar proteins were prepared. The SNF fraction reacted like the MSF fraction inasmuch as more of the titin protein doublet was present on SDS-gels as the NaCl concentration increased from 0.30M to 0.80M. That increased presence of titin as NaCl concentration increased was the case for each of the alkaline phosphate treatments. In

addition, Figures 7 and 8 show that the NaCl+10mM phosphates were more effective than NaCl alone in extracting titin into the SNF fraction because more titin was present on SDS-gels of the SNF fractions from each of the phosphate treatments than on SDS-gels of the SNF fraction from NaCl alone. If the 10mM phosphate treatments are compared on the basis on the intensity of the titin protein doublet on SDS-gels, NaCl+10mM PP extracted more titin in the SNF protein fraction than either NaCl+10mM TPP or NaCl+10mM SPG. Furthermore, NaCl+10mM TPP extracted more titin from myofibrils into the SNF fraction than NaCl+10mM SPG. The presence of nebulin on SDS-gels of the SNF fractions increased as the NaCl concentration increased for all treatments. The phosphate treatments appeared to extract more nebulin into the SNF protein fractions than NaCl alone; however, the differences between treatment with NaCl+10mM phosphate and NaCl alone were not as pronounced as it was with titin. In addition, no differences in the ability of the three phosphate treatments to remove nebulin from beef muscle tissue into SNF protein fractions was observed on SDS-gels in this study.

The 3.2% SDS-gel electrophoretograms in Figures 9 and 10 show the MFF protein fraction. These are the purified myofibrillar proteins from treated beef STM after removal of proteins into the MSF and SNF fractions. The decreased

titin band intensity at the higher NaCl concentrations, particularly when 10mM PP was combined with NaCl, pointed out that the greater amounts of titin detected in the 3.2% SDS-gels of the MSF and SNF fractions were actually due to the extraction effects of the four treatment groups rather than to the presence of more protein in the experimental samples. Because NaCl+10mM PP extracted the most titin into MSF and SNF fractions from STM, it showed the most decrease in titin band intensity as NaCl concentration increased. Nebulin was present in the MFF fractions of three treatment combinations: 0.30M NaCl, 0.30M NaCl+10mM TPP and 0.30M NaCl+10mM SPG. Thus, a majority of nebulin was either extracted into the MSF and SNF fractions or degraded by the other NaCl and NaCl+10mM phosphate treatments.

Figures 11, 12 and 13, 14 are the 12% SDS-gel electrophoretograms of the MSF and SNF protein fractions, respectively. These SDS-gels detected both sarcoplasmic and myofibrillar proteins that had molecular weights less than myosin heavy chain (MHC). The primary myofibrillar protein change caused by NaCl and NaCl+10mM phosphate treatments was the increased amount of MHC detected by the 12% SDS-gels as the NaCl concentration increased. That result was consistent for both NaCl and NaCl+10mM phosphate treatments and with both the MSF and SNF fractions. The greater amount of MHC detected at the higher NaCl concentrations for all

NaCl and NaCl+10mM phosphate treatments was expected because those treatments removed significant portions of beef myofibril A-bands (Figures 1-4). Also, myofibrillar proteins such as M-line protein and myosin light chain-1 (MLC-1) were observed in greater amounts on 12% SDS-gels at the higher NaCl concentrations of the NaCl and NaCl+10mM phosphate treatments for both the MSF and SNF fractions.

Each of the NaCl+10mM phosphate treatments extracted more MHC, M-line protein and MLC-1 into the MSF and SNF fractions than NaCl alone. Among the three NaCl+10mM phosphate treatment types, NaCl+10mM PP was the most effective, as judged by the intensity of protein bands on SDS-gels, in extracting MHC and M-line protein from beef STM and into the MSF protein fraction. Solutions containing NaCl+10mM SPG were the least effective at extracting these proteins. That result was anticipated because NaCl+10mM PP extracted more protein from the A-bands of irrigated myofibrils than either NaCl+10mM TPP or NaCl+10mM SPG. Conversely, SDS-gels of the SNF fraction (Figures 13, 14) revealed little difference in the ability of the different NaCl+10mM phosphate treatment types to extract MHC, M-line protein and MLC-1 into the SNF protein fraction.

The 12% SDS-gel electrophoretograms of the MFF protein fraction in Figures 15 and 16 show the decreased amounts of detectable MHC, and M-line protein, especially for the three

NaCl+10mM phosphate treatment types, as a result of the increased presence of those proteins in the MSF and SNF fractions. Because solutions containing only NaCl extracted relatively small amounts of MHC and M-line protein into the MSF and SNF fractions, little decrease in the band intensity of those proteins was observed on the SDS-gels of the MFF fraction as the NaCl concentration increased from 0.30M to 0.80M NaCl.

Effect of NaCl or NaCl+10mM phosphate on WHC

Means for percentage cooked yields, used as a measure of WHC for beef STM incubated with various NaCl or NaCl+10mM phosphate solutions, are presented in Table 2. Percentage cooked yields for beef STM increased significantly ($P<0.05$) as NaCl concentration increased from 0.30M to 0.80M NaCl within each of the four treatment types. At each NaCl concentration, the presence of 10mM phosphate in the NaCl solutions generally increased beef tissue WHC when compared to solutions containing NaCl alone. This was especially true for the NaCl solutions containing 10mM PP or 10mM TPP. Although NaCl+10mM SPG solutions produced significantly ($P<0.05$) higher WHC values for beef STM than NaCl alone at the NaCl concentrations of 0.30M and 0.47M, NaCl+10mM SPG did not produce higher WHC values than NaCl alone at 0.64M and 0.80M NaCl. Solutions containing NaCl+10mM PP generally

yielded the highest WHC values and, indeed, NaCl+10mM PP yielded significantly ($P<0.05$) higher WHC values than NaCl+10mM TPP at the NaCl concentrations of 0.47M and 0.80M. The ability of NaCl+10mM phosphate to increase the WHC of beef STM above NaCl alone can be shown inasmuch as WHC values all of the 0.30M NaCl+10mM phosphate solutions were actually higher than 0.47M NaCl alone. WHC values for all of the 0.47M NaCl+10mM phosphate solutions were equal to, or greater than 0.64 NaCl, and the WHC values for the 0.64M NaCl+10mM phosphate solutions were nearly equal to, or greater than the WHC values for solutions containing 0.80M NaCl.

DISCUSSION

Myofibril swelling and structural changes

Paterson et al. (1987) used phase-contrast microscopy to study the swelling and structural changes of isolated beef myofibrils subjected to NaCl solutions of varying concentrations (0.10M = ~.45%; 0.40M = ~1.70%; 0.70M = ~3.0%; and 1.0M = ~4.5% NaCl) with and without 10mM PP (= ~.3%). In that prior study, the major changes in myofibril swelling and structure began at 0.40M NaCl, in the presence of 10mM PP, and at 0.70M NaCl in the absence of 10mM PP. The present experiment was designed then to more closely examine that NaCl concentration range where the major changes had occurred. The NaCl concentrations included: 0.30M (~1.4%), 0.47M (~2.1%), 0.64M (~2.8%) and 0.80M (~3.5%). These beef tissue NaCl concentrations are typical of those found in the wide variety of processed meat products available to today's consumer. In addition to PP, TPP and SPG were also added to NaCl solutions and studied because both are food grade phosphates used in processed meat products. Myofibril swelling and structural changes exhibited by myofibrils treated with NaCl or NaCl+10mM PP closely mirrored the results reported by Paterson et al. (1987). The addition of 10mM PP to NaCl solutions significantly improved myofibril swelling and myofibrillar

protein extraction, inasmuch as more of the A-bands were removed by NaCl+10mM PP. The effectiveness of PP was in agreement with the results reported by Offer and Trinick (1983) and Voyle et al. (1984).

Lewis et al. (1986) used electron microscopy to study the effects of NaCl and phosphates on different pork and beef muscles. Electron micrographs revealed that A-/I-bands were the most resistant regions to extraction by NaCl and phosphate. Solutions containing PP or TPP produced some dissociation of actomyosin in beef heart and forequarter muscle but not to the extent observed in the present study. Lewis et al. (1986) studied cooked myofibrils rather than raw, as used in the present study, so direct comparisons of phosphate treatments should not be made.

In the present study, NaCl solutions containing 10mM TPP were as effective at increasing beef myofibril swelling, above NaCl alone, as were solutions containing NaCl+10mM PP. Conversely, NaCl solutions containing 10mM SPG were less effective at increasing myofibril swelling than either NaCl+10mM PP or NaCl+10mM TPP, but NaCl+10mM SPG was more effective than NaCl alone. This swelling data is somewhat confusing in view of the myofibril structural changes shown in Figures 1-4. It was clear that NaCl+10mM PP solutions extracted far more protein from myofibril A-bands than did NaCl+10mM TPP solutions, and yet, there was no difference in

myofibril swelling between the two phosphate treatments. However, NaCl+10mM TPP did extract more protein from myofibrils than NaCl+10mM SPG and both extracted more protein than NaCl alone. But, unlike the PP/TPP comparison, the protein extraction differences between NaCl+10mM TPP, NaCl+10mM SPG and NaCl alone did seem to be followed by corresponding differences in myofibril swelling between the three treatments.

Perhaps there is a critical amount of myofibrillar protein or type of myofibrillar protein that needs to be extracted from myofibrils in order to produce maximal myofibril swelling. Consequently, the extraction of myofibrillar protein beyond this critical amount will not produce any further increases in myofibril swelling. This would explain that even though NaCl+10mM PP extracted much more protein than NaCl+10mM TPP, NaCl+10mM TPP extracted the critical amount of protein necessary to achieve maximal myofibril swelling. Thus, NaCl+10mM SPG and to a greater extent NaCl alone, did not extract the critical amount or type of myofibrillar protein and thus produced decreased amounts of beef myofibril swelling.

SDS-PAGE

In the present study, protein fractions identical to those studied in the previous work by Paterson et al. (1987)

were evaluated with SDS-PAGE. Results of the SDS-gels from NaCl and NaCl+10mM PP treated beef muscle in this study were very similar to results reported by Paterson et al. (1987). Increasing the NaCl concentration increased the presence of titin, nebulin, MHC and M-line protein on SDS-gels of the MSF and SNF fractions and the addition of 10mM PP to NaCl solutions further increased the occurrence of these myofibrillar/cytoskeletal proteins on MSF and SNF fraction SDS-gels. Just as previously reported by Paterson et al. (1987), NaCl+10mM PP preferentially extracted protein from the A-/I-band junctions while NaCl solutions extracted protein from A-band centers. The consequence of this extraction difference was that more titin was present on MSF and SNF SDS-gels from NaCl+10mM PP treated beef muscle. This result gives further evidence that the location for the majority of titin is in the A-/I-band region of myofibrils as suggested by Wang et al. (1979; 1984) and LaSalle et al. (1983).

Although the presence of 10mM TPP or 10mM SPG in NaCl solutions increased the occurrence of myofibrillar / cytoskeletal proteins on MSF and SNF SDS-gels above NaCl alone, neither 10mM TPP or 10mM SPG increased the presence of myofibrillar/cytoskeletal protein, especially titin, on MSF or SNF SDS-gels to the extent produced by 10mM PP. Indeed, 10mM TPP extracted protein from the A-/I-band

junctions of myofibrils only at the 0.80M NaCl concentration while 10mM SPG did not extract protein from A-/I-band junctions at any of the four NaCl concentrations

Wang et al. (1984) showed that titin was soluble at the ionic strength created by the Guba-Straub solution (0.30M KCl, 0.15M K phosphate pH 6.7). Trout and Schmidt (1986a) have reported that as phosphate chain length increased, the dissociation of the phosphate in solution decreased, and thus, the ionic strength of that solution is decreased. According to Trout and Schmidt (1986a), the average chain length for the phosphates used in this study were: PP = 2.0, TPP = 3.0 and SPG = 20.8, and the dissociation of these phosphates in a .45% phosphate solution (~10mM) is: PP = 88.4%, TPP = 81.2% and SPG = 37.99%. Therefore, at the same NaCl concentration, solutions containing 10mM PP would have the highest ionic strengths, solutions with 10mM TPP would have an intermediate ionic strengths and solutions containing 10mM SPG would have the lowest ionic strengths. Trout and Schmidt (1986b) concluded that increases in ionic strength and pH are the two most important contributions of phosphates for improving the functional properties of meat, of which, myofibrillar protein extraction is one such property. The pH of all solutions was adjusted to 5.5 so ionic strength differences between the NaCl, NaCl+10mM PP, NaCl+10mM TPP and NaCl+10mM SPG treatments may have played a

role in creating the SDS-PAGE differences detected between the treatments.

Water-holding capacity

The WHC data (Table 2) indicated that the combination of NaCl with 10mM phosphate generally increased WHC values more than NaCl alone. NaCl+10mM PP was slightly more effective at increasing WHC than NaCl+10mM TPP. In turn, NaCl+10mM TPP was more effective than NaCl+10mM SPG. These results are in agreement with Shults et al. (1972) and Shults and Wierbicki (1973) who reported that phosphates combined with NaCl increased WHC more than NaCl alone, and PP was the most effective phosphate for increasing meat WHC.

Lewis et al. (1986) reported that the presence of PP or TPP in a 4% NaCl solution increased the WHC of beef and pork muscle more than solutions containing only 4% NaCl. They attributed that increased WHC to two factors: (1) the ability of phosphates to increase the pH of a meat system, and (2) the ability of phosphates to increase the dispersion of myofibrillar proteins. Although increased pH due to the presence of phosphates may be a factor in improving meat WHC, this study and previous work (Paterson et al., 1987) show that the addition of phosphates, particularly PP and TPP, to NaCl solutions definetly improved beef muscle WHC even though the NaCl+10mM phosphate solutions all had the

same pH (5.5). Thus, some other factor(s), attributable to phosphates, must be involved in determining meat WHC.

Phase-contrast micrographs and SDS-gels from the present study clearly showed that NaCl+10mM phosphate treatments, particularly PP, extracted more myofibrillar protein from beef muscle than NaCl alone and those same NaCl+10mM phosphate treated samples had higher WHC values. Thus, in partial agreement with the conclusions of Lewis et al. (1986), myofibrillar protein extraction does appear to be a factor that associates phosphates with increased meat WHC. Trout and Schmidt (1984, 1986b) concluded that increased ionic strength was the most important factor by which phosphates increased meat WHC. As previously mentioned, the NaCl+10mM PP and NaCl+10mM TPP solutions had higher ionic strengths then either NaCl+10mM SPG or NaCl alone. Because NaCl+10mM PP and NaCl+10mM TPP solutions produced the highest WHC values, it would appear then that the ionic strength of treatment solutions played an important role in determining beef muscle WHC.

Conclusions

Overall, the addition of 10mM phosphate to NaCl solutions increased beef myofibril swelling, enhanced myofibrillar protein extraction and improved beef muscle WHC more than solutions containing only NaCl. The three

different phosphates affected myofibril swelling, myofibrillar protein extraction and beef muscle WHC differently, but as a generalization, the different phosphates increased the functional properties of myofibril swelling, protein extraction and WHC in the following order: PP > TPP > SPG. These results are in accordance with those reported by Shults et al. (1972) and Trout and Schmidt (1984, 1986b). In the present study, 10mM PP combined with NaCl was particularly effective in extracting myofibrillar proteins, especially titin, from beef muscle. Paterson et al. (1987) also reported that NaCl+10mM PP effectively extracted titin from beef muscle. In addition, Offer and Trinick (1983), Voyle et al. (1984) and Lewis et al. (1986) observed that PP, when combined with NaCl, effectively extracted myofibrillar proteins from rabbit, pork and beef muscle, respectively.

Results indicated that the extraction of a critical amount of myofibrillar protein or a critical type of myofibrillar protein was necessary for maximal myofibril swelling and maximal beef muscle WHC to occur. Evidence from SDS-gels indicated that the high molecular weight cytoskeletal protein titin may be the critical protein of which extraction was necessary to gain increased myofibril swelling and increased beef muscle WHC.

Table 1. Mean myofibril swelling values[1] of beef muscle irrigated with various NaCl and NaCl+10mM phosphate solutions[2,3]

NaCl Concentration	Phosphate Treatment			
	NaCl	NaCl+PP	NaCl+TPP	NaCl+SPG
0.30M NaCl	102.4^{ax}	110.4^{ay}	109.0^{ay}	108.6^{ay}
0.47M NaCl	110.2^{bx}	125.0^{bz}	127.1^{bz}	120.8^{by}
0.64M NaCl	124.0^{cx}	140.6^{cz}	139.7^{cz}	133.2^{cy}
0.80M NaCl	138.3^{dx}	146.0^{dy}	146.3^{dy}	139.4^{dx}

[1] n=20.

[2] 100% = No increase in myofibril diameter.

[3] SE = 2.3.

^{abcd}Means that bear unlike superscripts in each column differ significantly ($P<0.05$).

^{xyz}Means that bear unlike superscripts in each row differ significantly ($P<0.05$).

Table 2. Mean percentage cooked yields[1] of beef muscle treated with various NaCl and NaCl+10mM phosphate solutions[2,3]

NaCl	Phosphate Treatment			
Concentration	NaCl	NaCl+PP	NaCl+TPP	NaCl+SPG
0.30M NaCl	93.1^{ax}	98.8^{ay}	100.2^{ay}	99.1^{ay}
0.47M NaCl	96.9^{bx}	109.0^{bz}	105.8^{by}	104.0^{by}
0.64M NaCl	105.3^{cx}	112.1^{cy}	110.7^{cy}	107.1^{cx}
0.80M NaCl	110.0^{dx}	121.3^{dz}	118.2^{dy}	111.2^{dx}

[1] n=20.

[2] 100% = Original tissue weight.

[3] SE = 1.7.

^{abcd}Means that bear unlike superscripts in each column differ significantly ($P<0.05$).

^{xyz}Means that bear unlike superscripts in each row differ significantly ($P<0.05$).

Fig. 1 - Phase-contrast micrographs of beef myofibrils after irrigation with various NaCl solutions.

Magnification = 2800x. 0 s = myofibril before irrigation; 180 s = myofibril after 180 s of irrigation

NaCl Irrigated Myofibrils

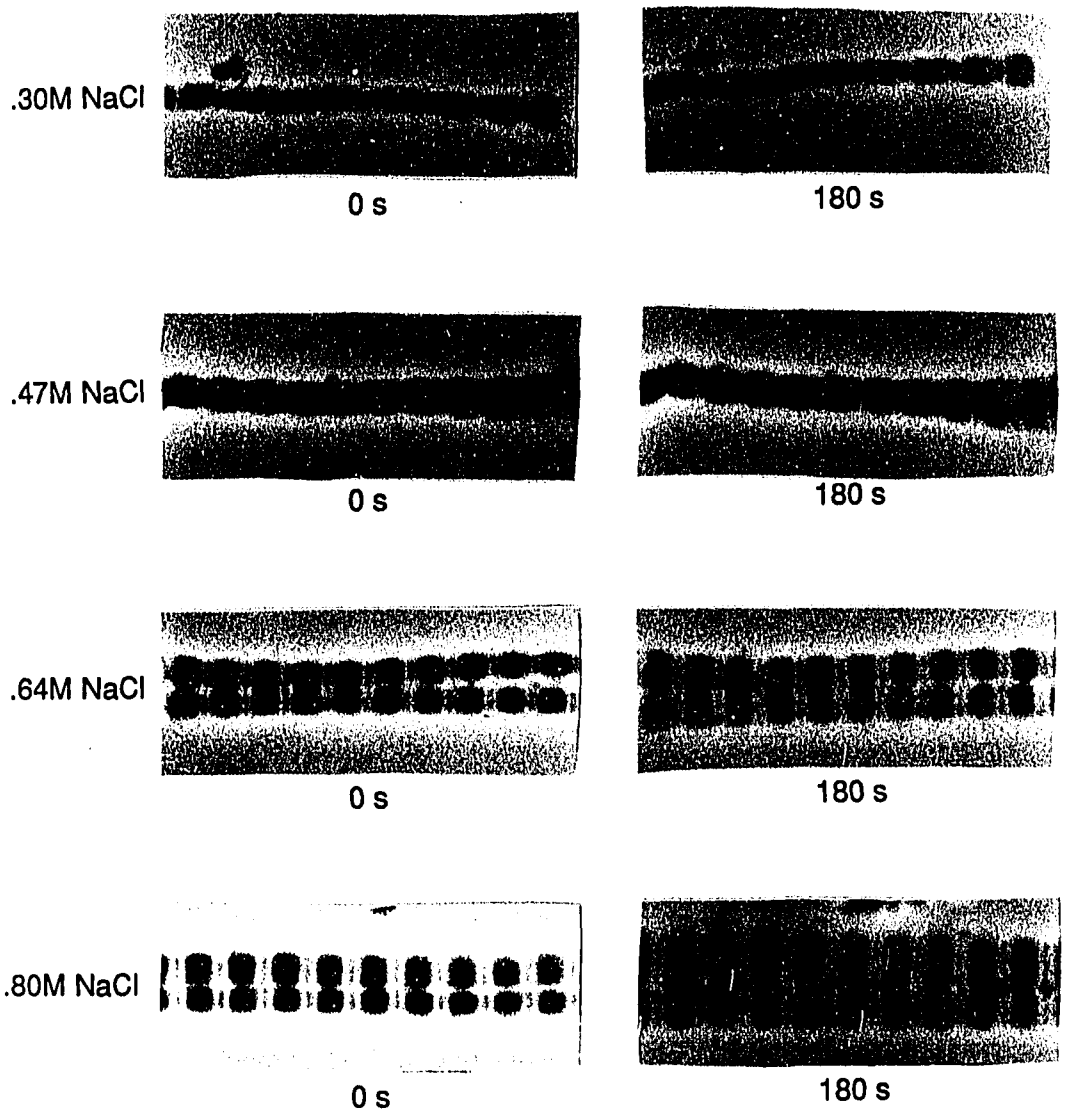


Fig. 2 - Phase-contrast micrographs of beef myofibrils after irrigation with various NaCl+10mM PP solutions.

Magnification = 2800x. 0 s = myofibril before irrigation; 180 s = myofibril after 180 s of irrigation

NaCl + 10mM Pyrophosphate Irrigated Myofibrils

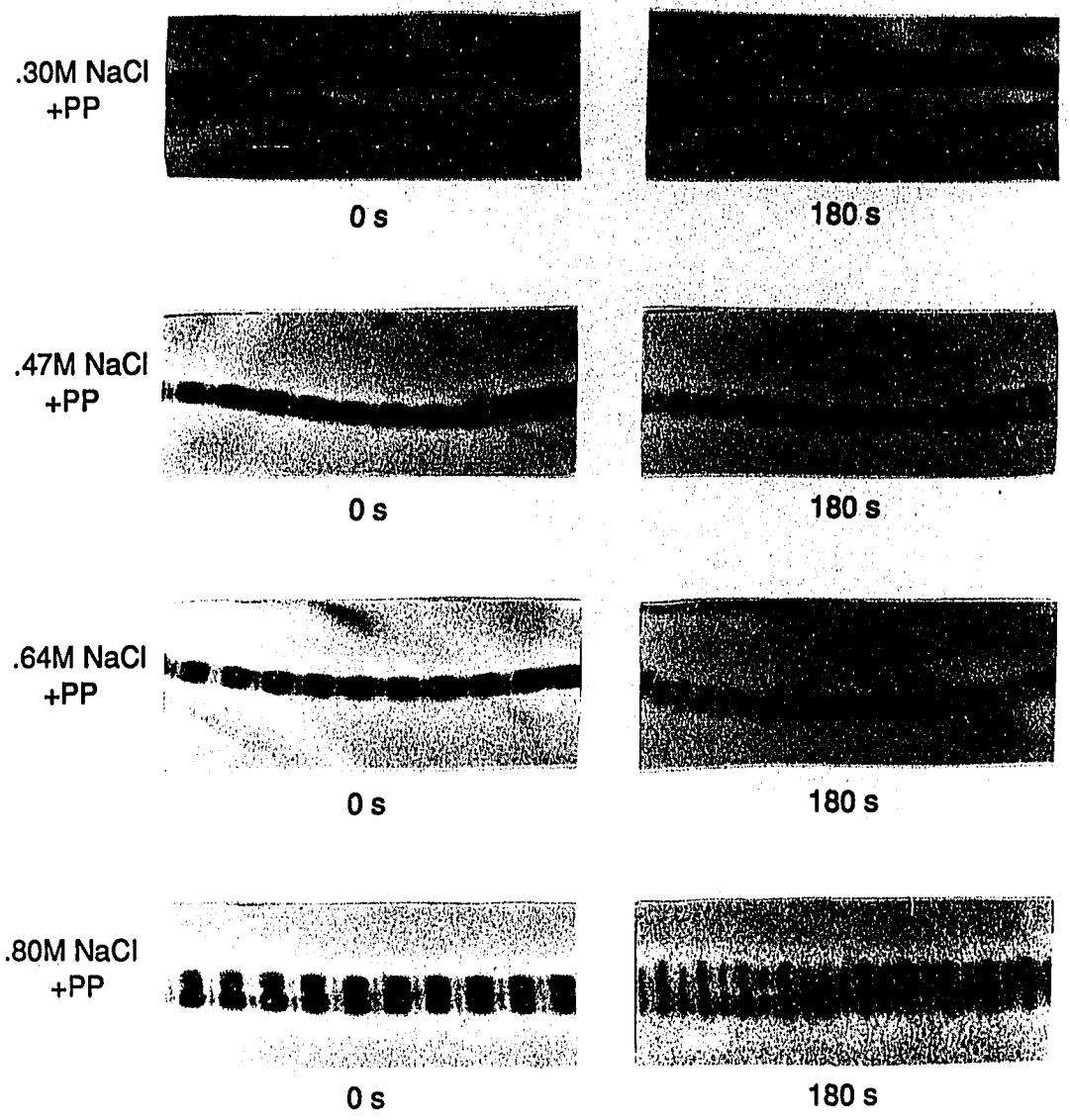


Fig. 3 - Phase-contrast micrographs of beef myofibrils after irrigation with various NaCl+10mM TPP solutions.

Magnification = 2800x. 0 s = myofibril before irrigation; 180 s = myofibril after 180 s of irrigation

NaCl + 10mM Tripolyphosphate Irrigated Myofibrils

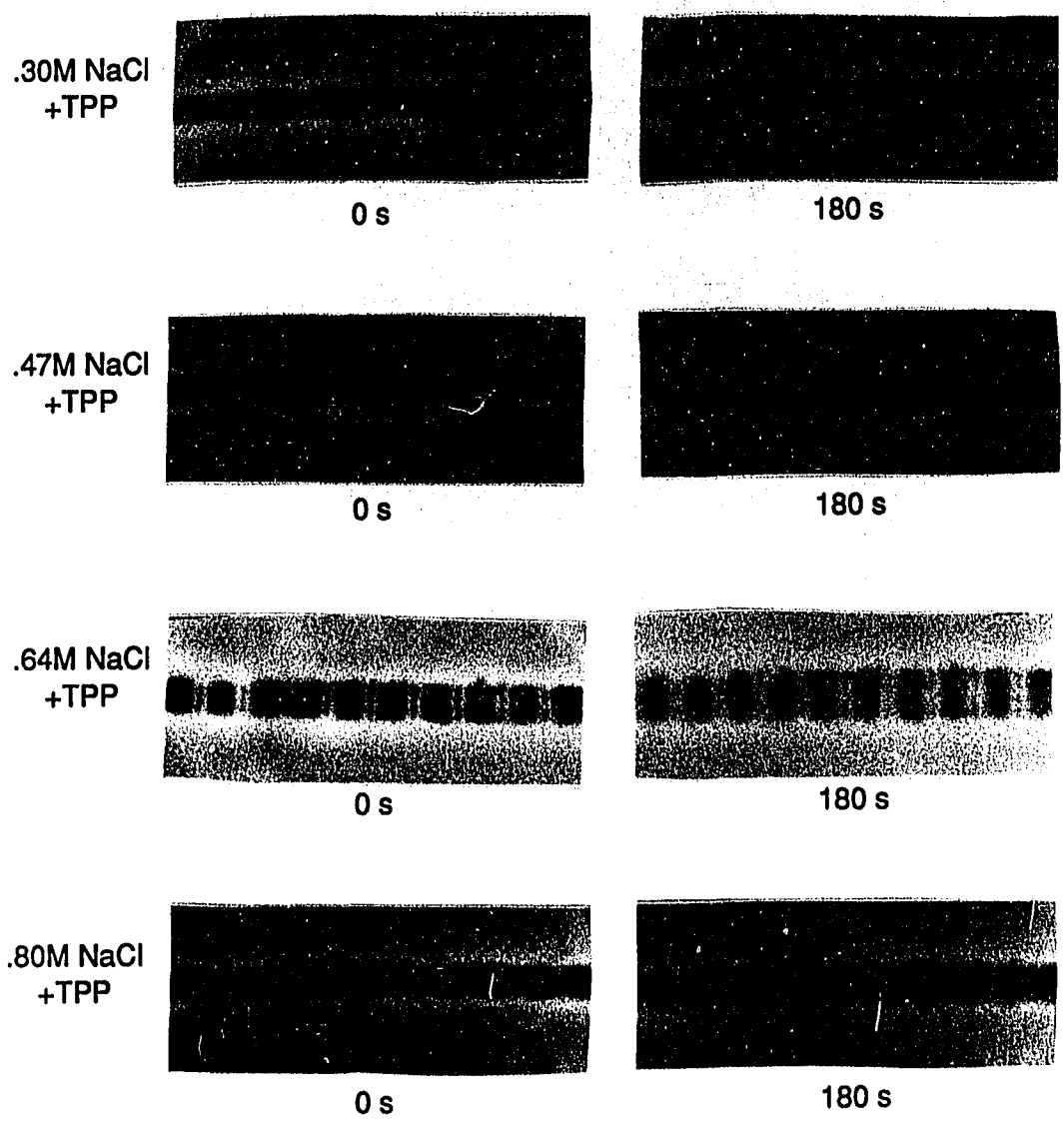


Fig. 4 - Phase-contrast micrographs of beef myofibrils after irrigation with various NaCl+10mM SPG solutions.

Magnification = 2800x. 0 s = myofibril before irrigation; 180 s = myofibril after 180 s of irrigation

NaCl + 10mM Sodium Polyphosphate (Glassy)
Irrigated Myofibrils

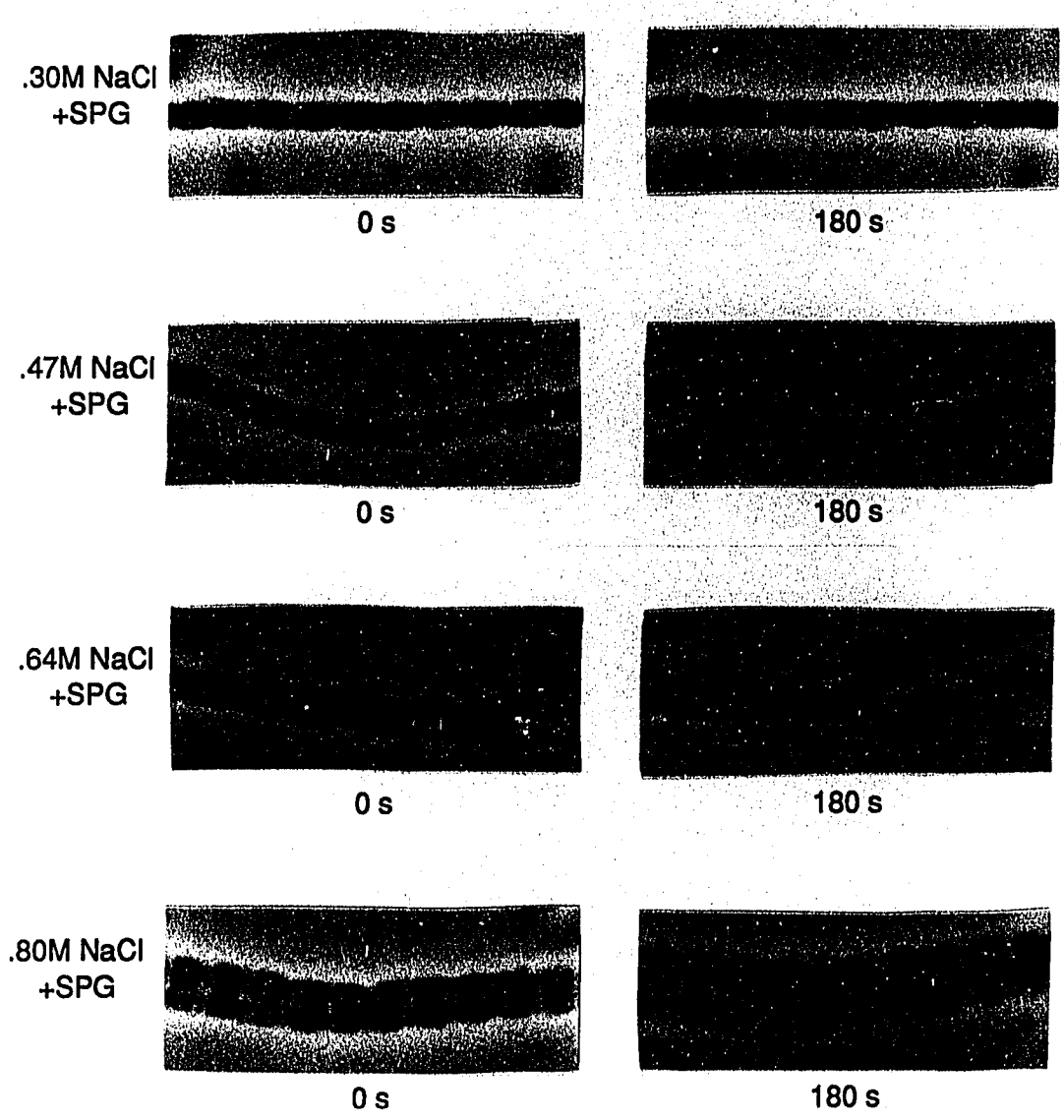


Fig. 5 - 3.2% SDS-gels (silver-stained) of beef muscle/NaCl/NaCl+10mM PP homogenates from beef sternomandibularis muscle after treatment with various NaCl and NaCl+10mM PP solutions

BEEF MUSCLE/NaCl/PP SOLUTION

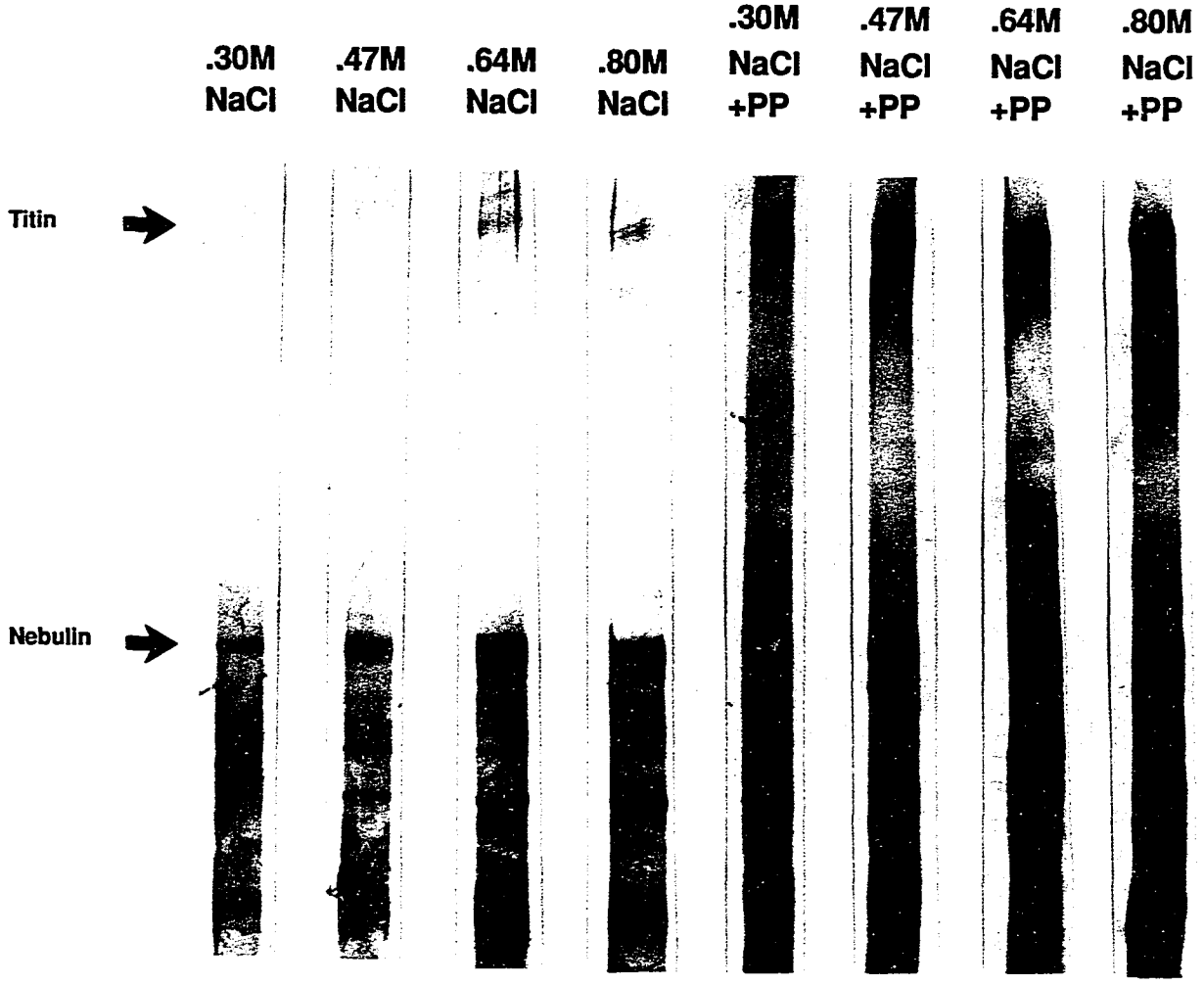


Fig. 6 - 3.2% SDS-gels (silver-stained) of beef/NaCl/TPP/SPG homogenates from beef sternomandibularis muscle after treatment with various NaCl+10mM TPP and NaCl+10mM SPG solutions

BEEF/NaCl/TPP/SPG SOLUTION

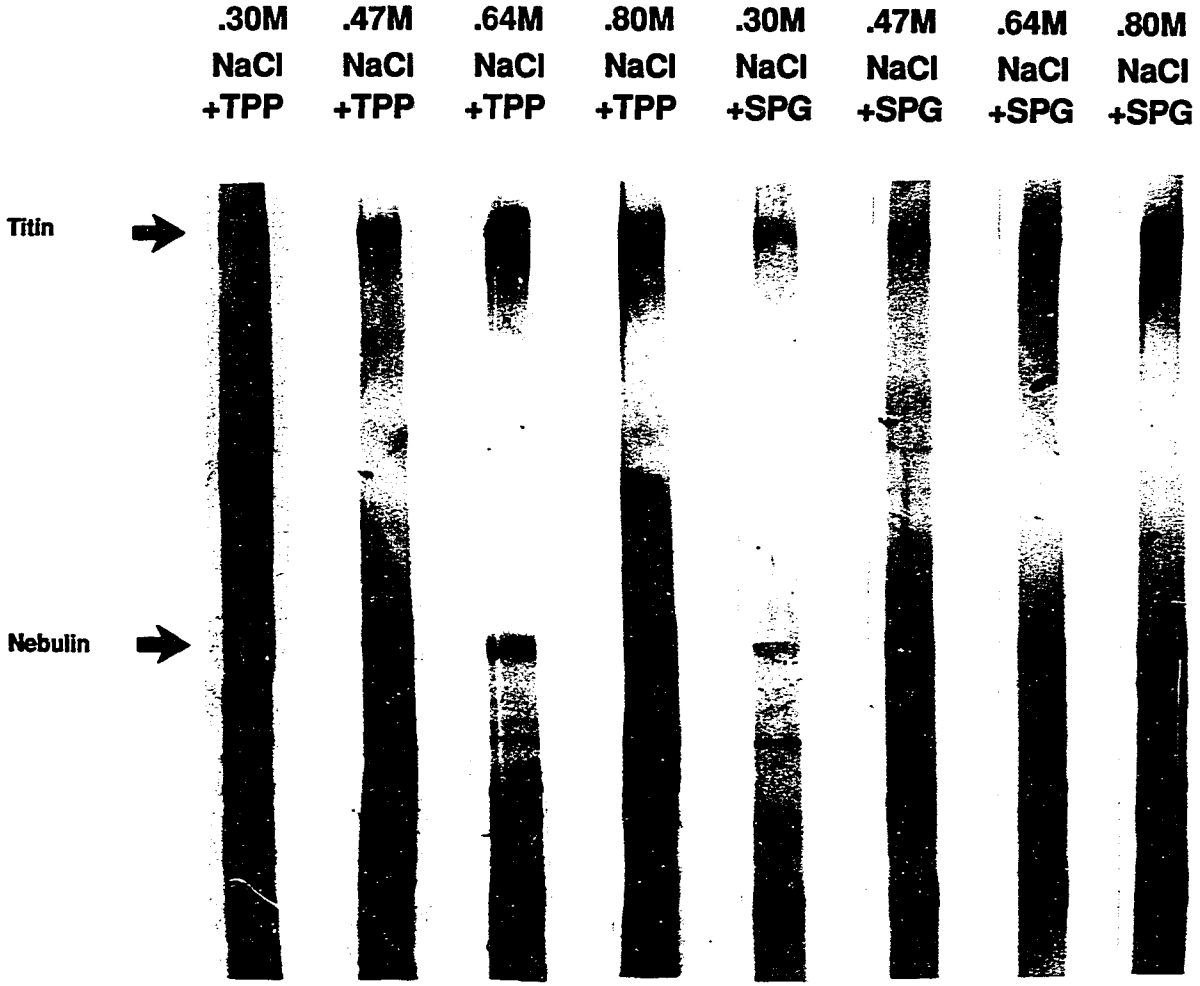


Fig. 7 - 3.2% SDS-gels (silver-stained) of supernatants from the myofibril protein purification procedure of beef sternomandibularis muscle after treatment with various NaCl and NaCl+10mM PP solutions

BEEF SUPERNATANT

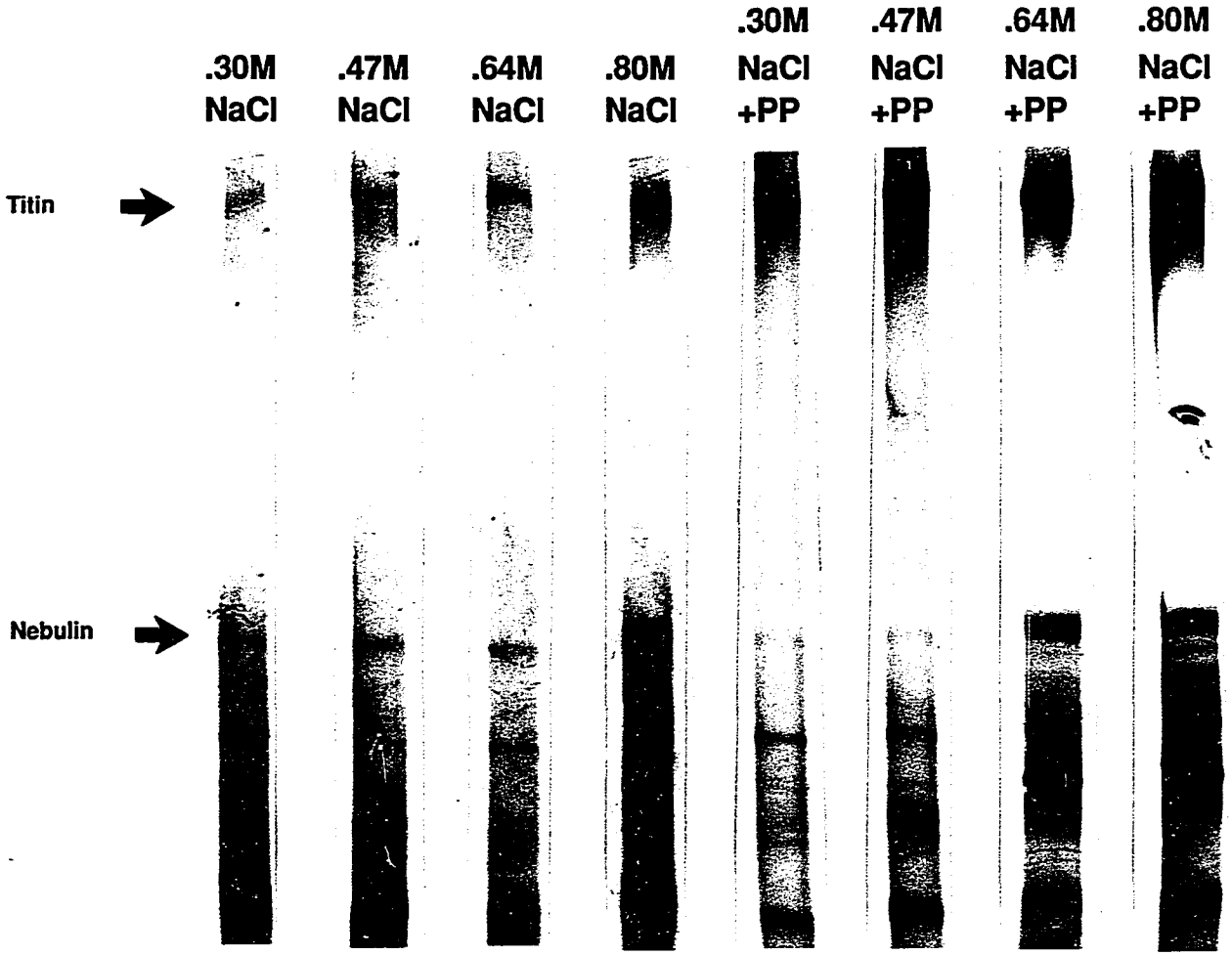


Fig. 8 - 3.2% SDS-gels (silver-stained) of supernatants from the myofibril protein purification procedure of beef sternomandibularis muscle after treatment with various NaCl+10mM TPP and NaCl+10mM SPG solutions

BEEF SUPERNATANT

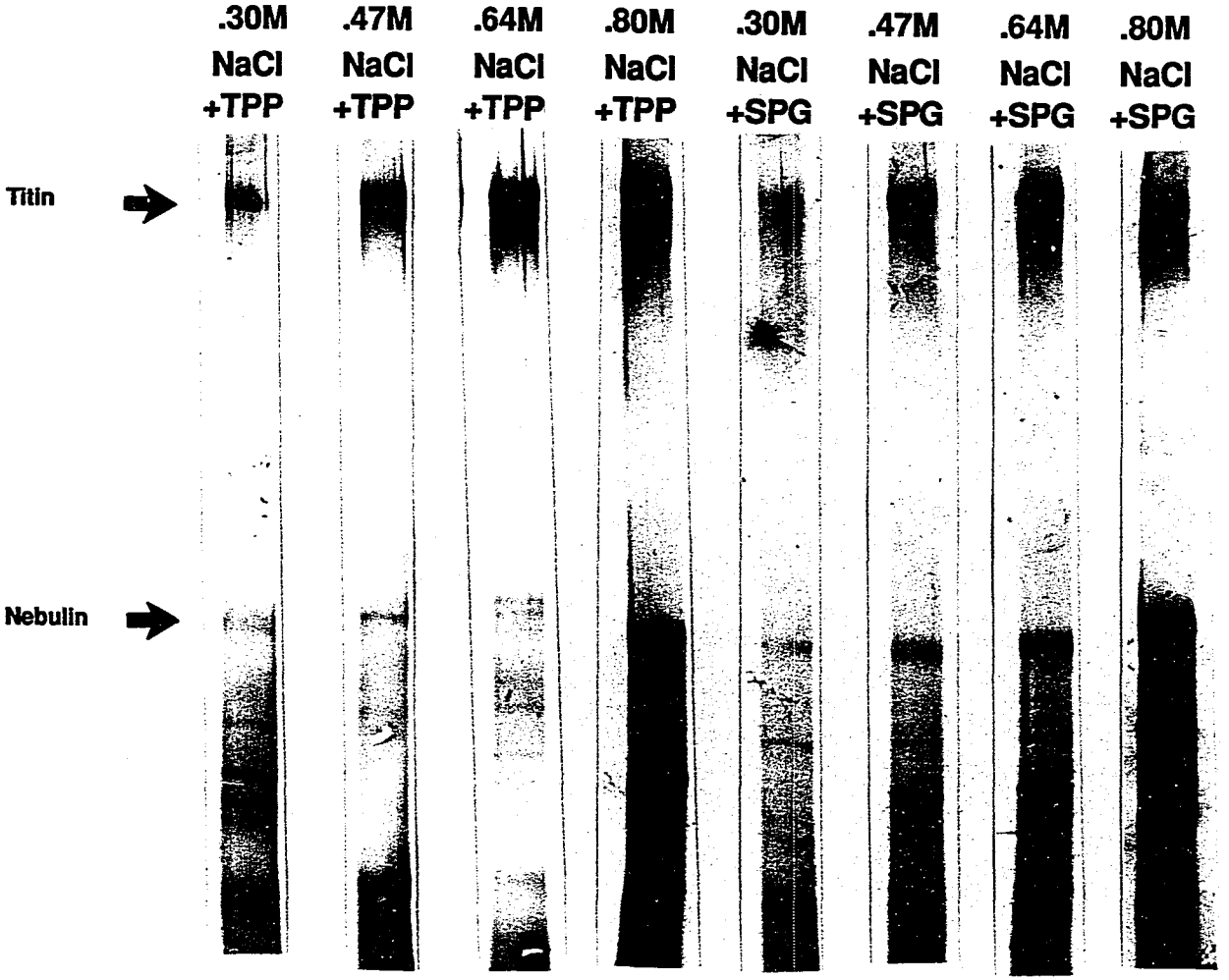


Fig. 9 - 3.2% SDS-gels (silver-stained) of purified myofibrillar/cyotskeletal proteins from beef sternomandibularis muscle after treatment with various NaCl and NaCl+10mM PP solutions

BEEF MYOFIBRILS

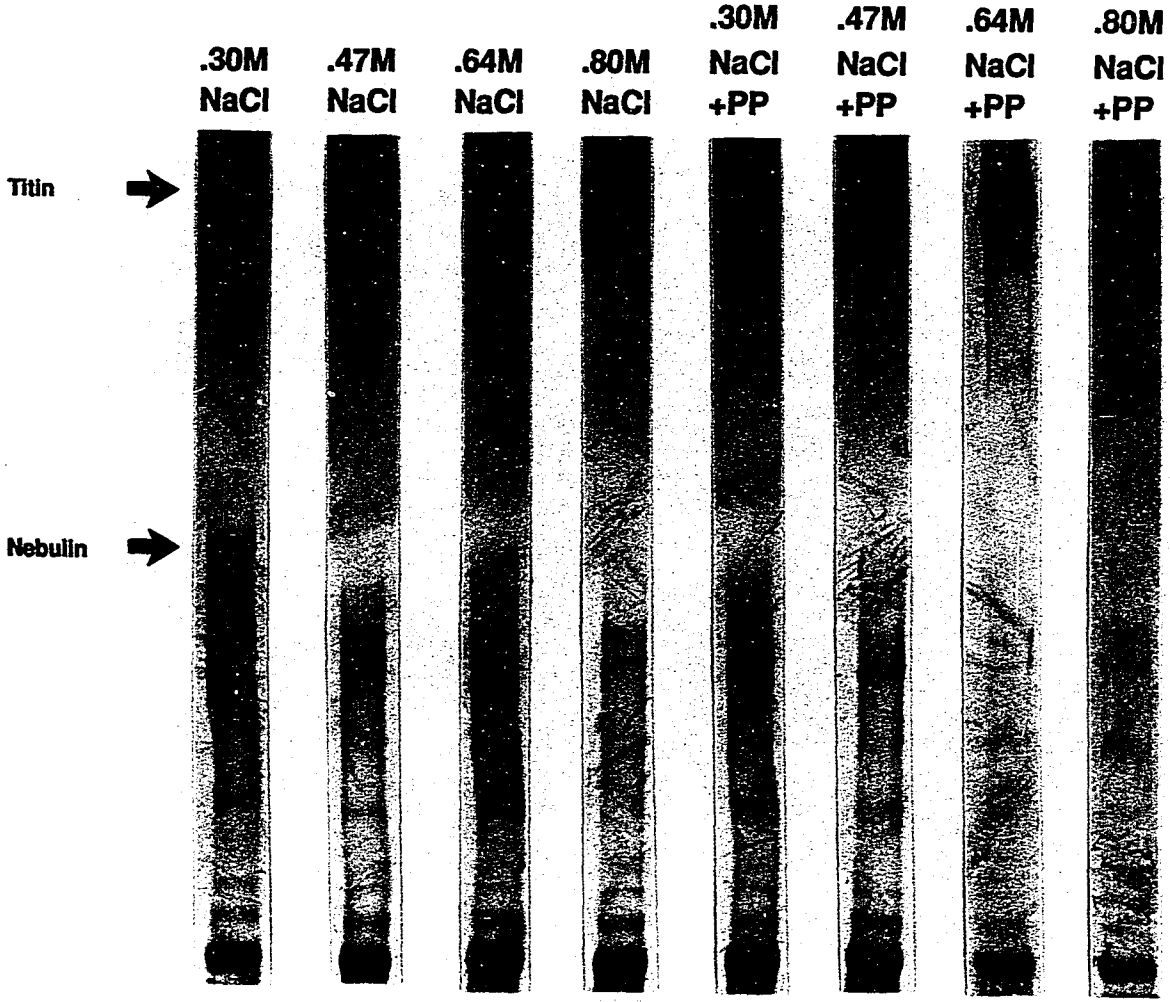


Fig. 10 - 3.2% SDS-gels (silver-stained) of purified myofibrillar/cyotskeletal proteins from beef sternomandibularis muscle after treatment with various NaCl+10mM TPP and NaCl+10mM SPG solutions

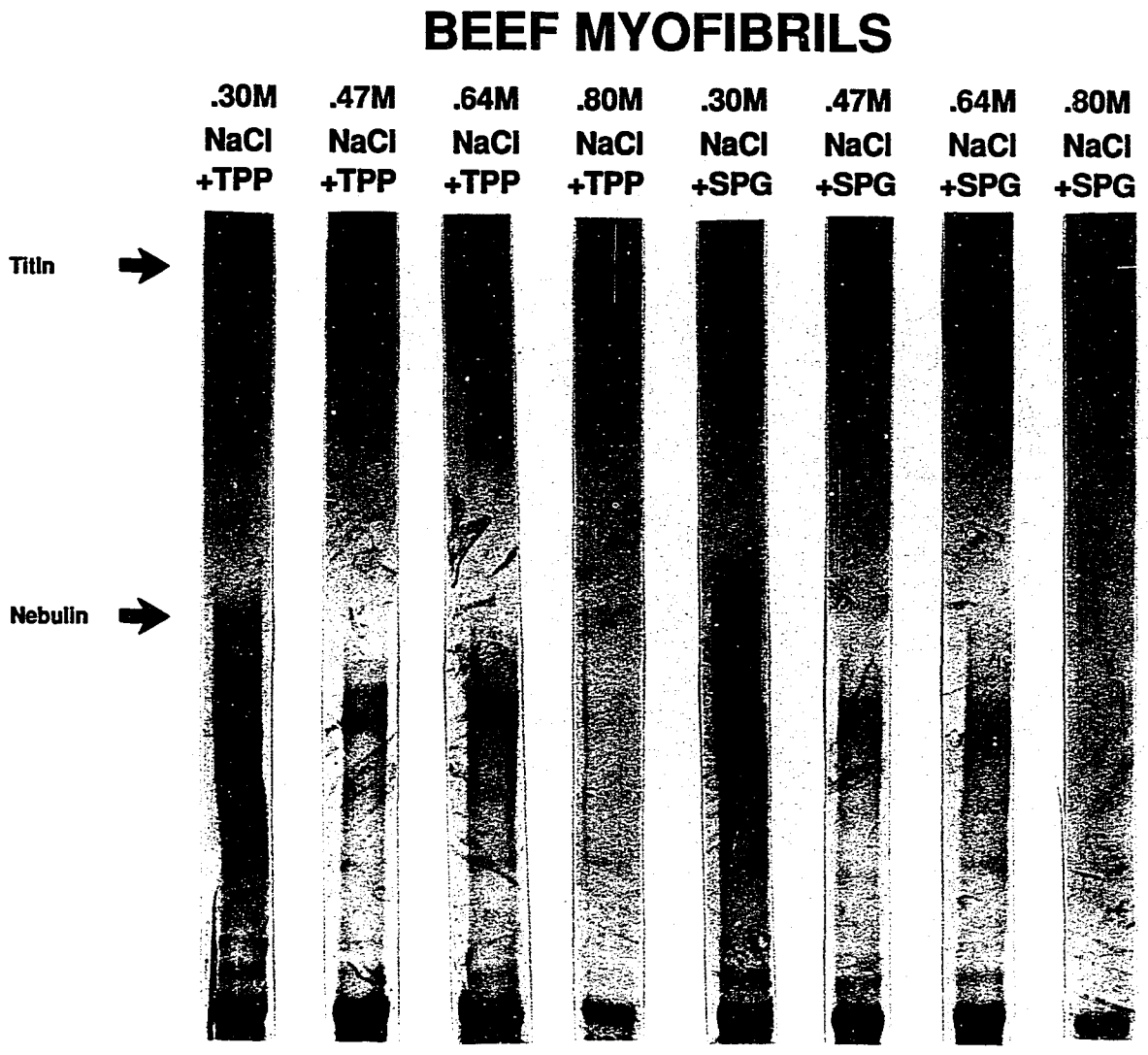

BEEF MYOFIBRILS
.30M NaCl +TPP
.47M NaCl +TPP
.64M NaCl +TPP
.80M NaCl +TPP
.30M NaCl +SPG
.47M NaCl +SPG
.64M NaCl +SPG
.80M NaCl +SPG
Titin
Nebulin

Fig. 11 - 12% SDS-gels (Coomassie blue-stained) of beef muscle/NaCl/NaCl+10mM PP homogenates from beef sternomandibularis muscle after treatment with various NaCl and NaCl+10mM PP solutions

BEEF MUSCLE/NaCl/PP SOLUTION

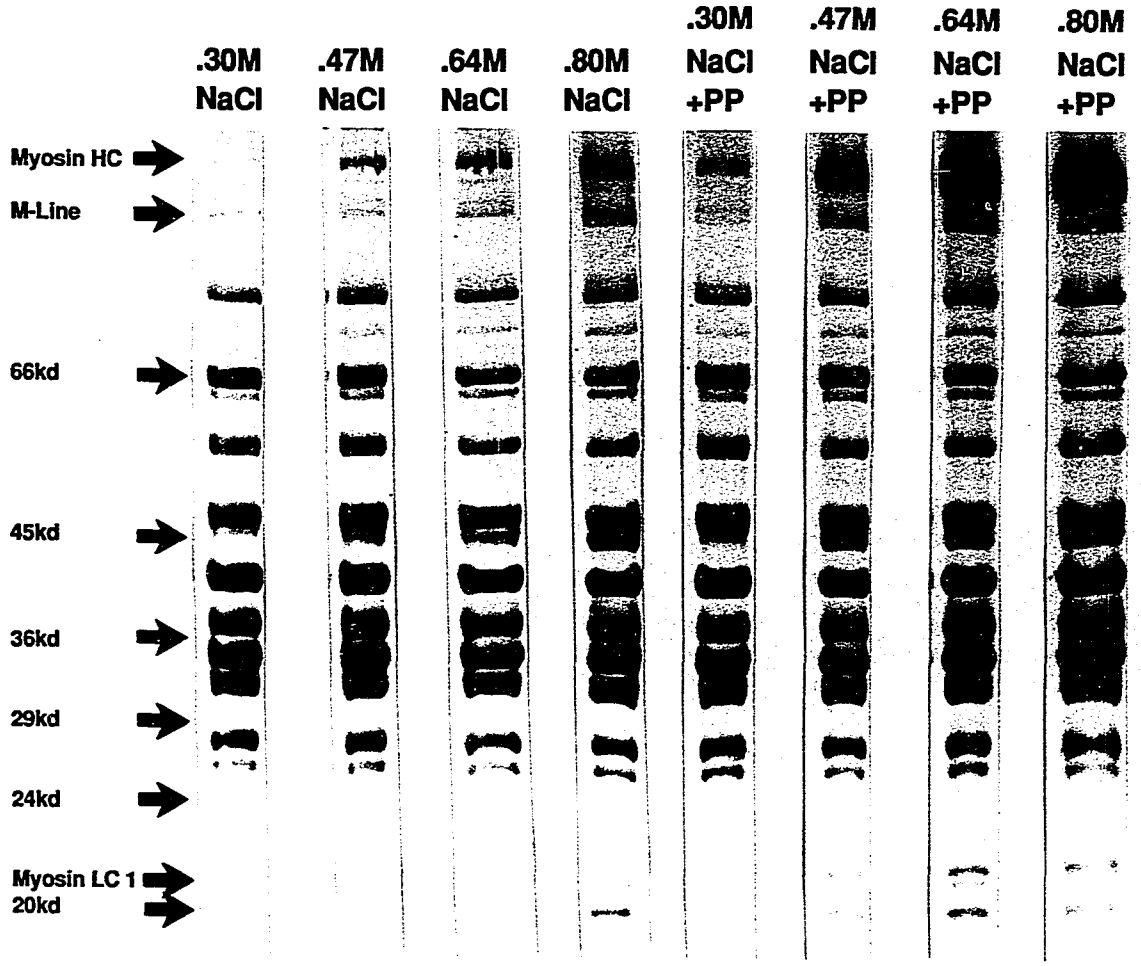


Fig. 12 - 12% SDS-gels (Coomassie blue-stained) of beef/NaCl/TPP/SPG homogenates from beef sternomandibularis muscle after treatment with various NaCl+10mM TPP and NaCl+10mM SPG solutions

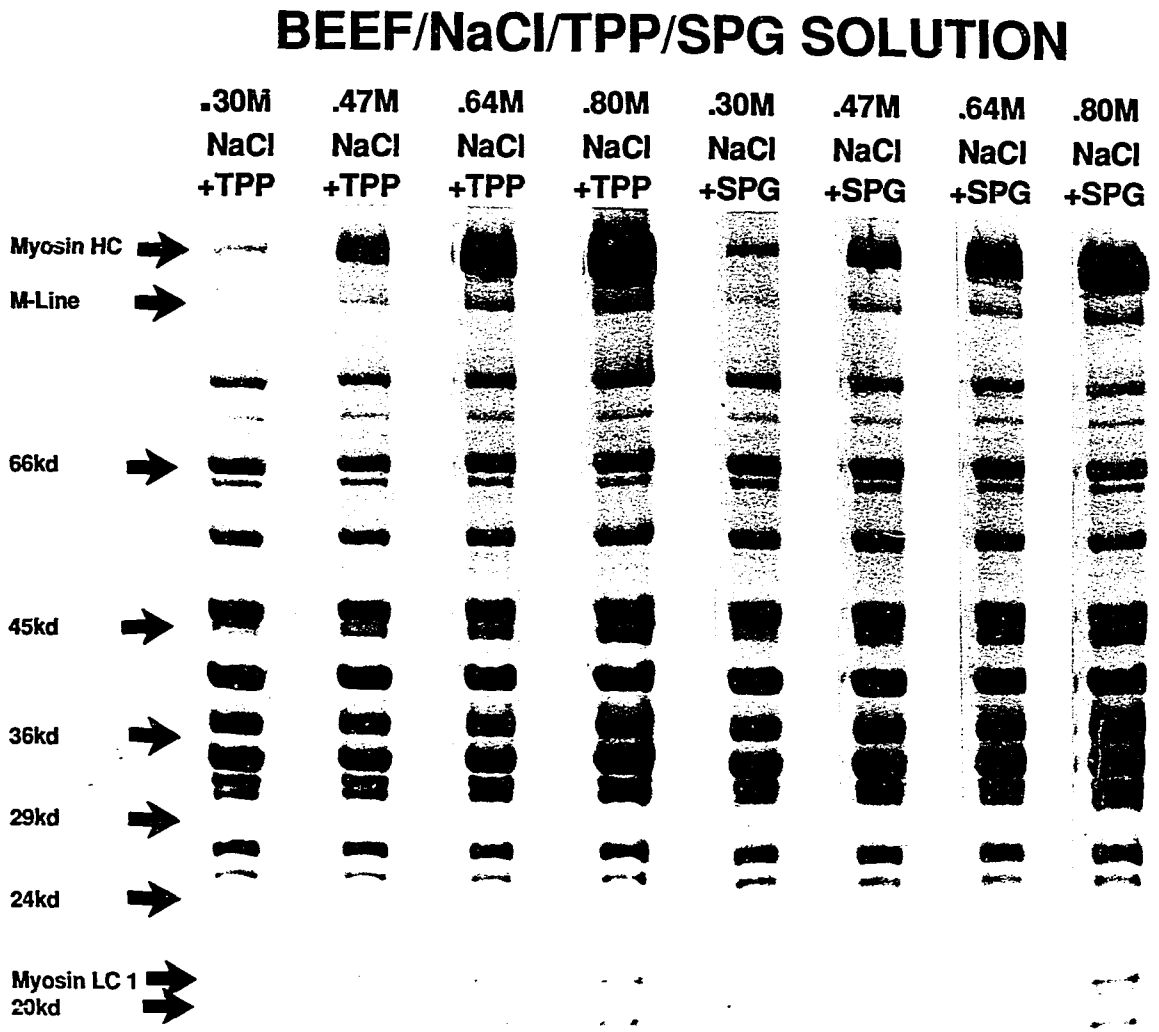

BEEF/NaCl/TPP/SPG SOLUTION
.30M NaCl +TPP
.47M NaCl +TPP
.64M NaCl +TPP
.80M NaCl +TPP
.30M NaCl +SPG
.47M NaCl +SPG
.64M NaCl +SPG
.80M NaCl +SPG
Myosin HC
M-Line
66kd
45kd
36kd
29kd
24kd
Myosin LC 1
20kd

Fig. 13 - 12% SDS-gels (Coomassie blue-stained) of supernatants from the myofibril protein purification procedure of beef sternomandibularis muscle after treatment with various NaCl and NaCl+10mM PP solutions

BEEF SUPERNATANT

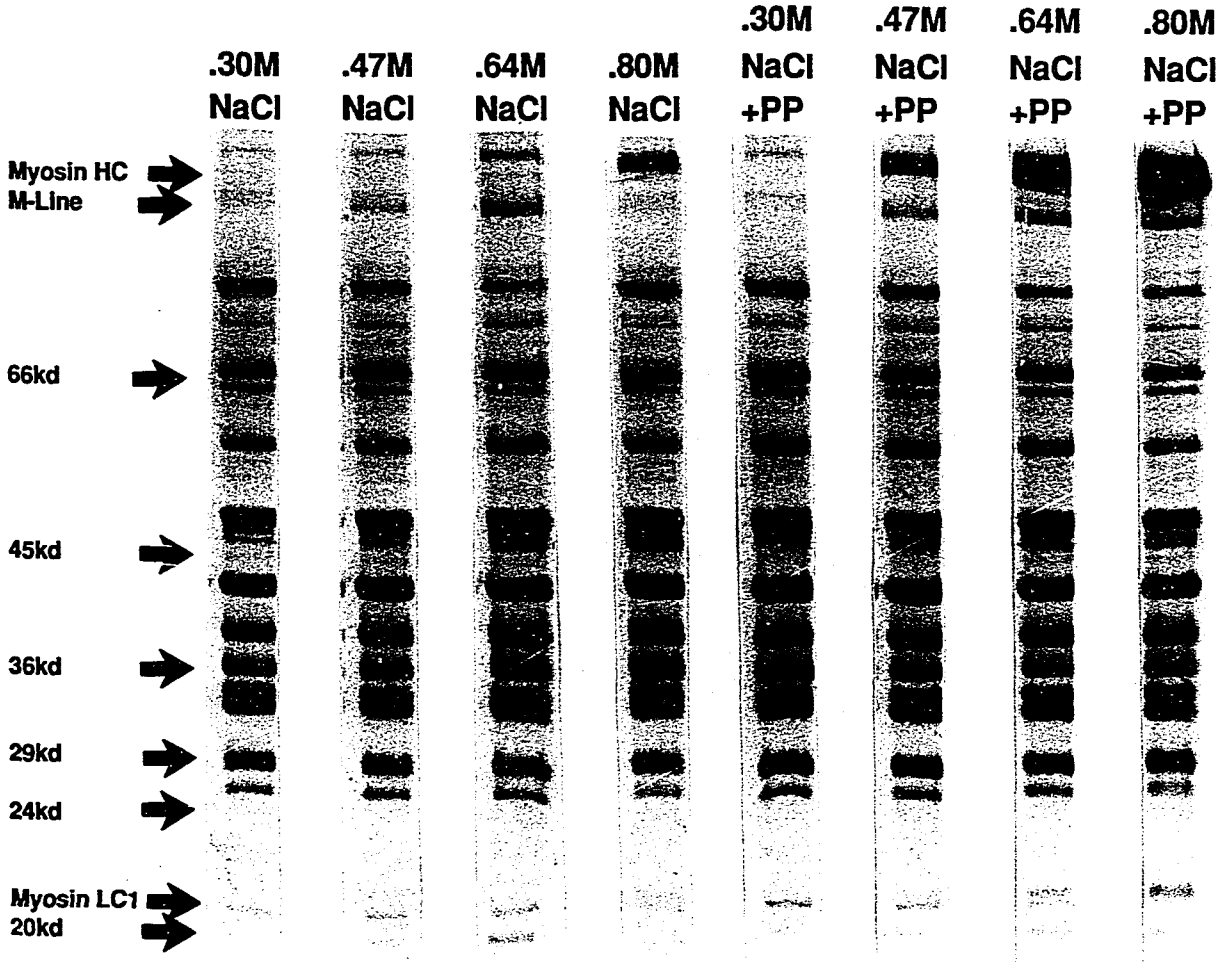


Fig. 14 - 12% SDS-gels (Coomassie blue-stained) of supernatants from the myofibril protein purification procedure of beef sternomandibularis muscle after treatment with various NaCl+10mM TPP and NaCl+10mM SPG solutions

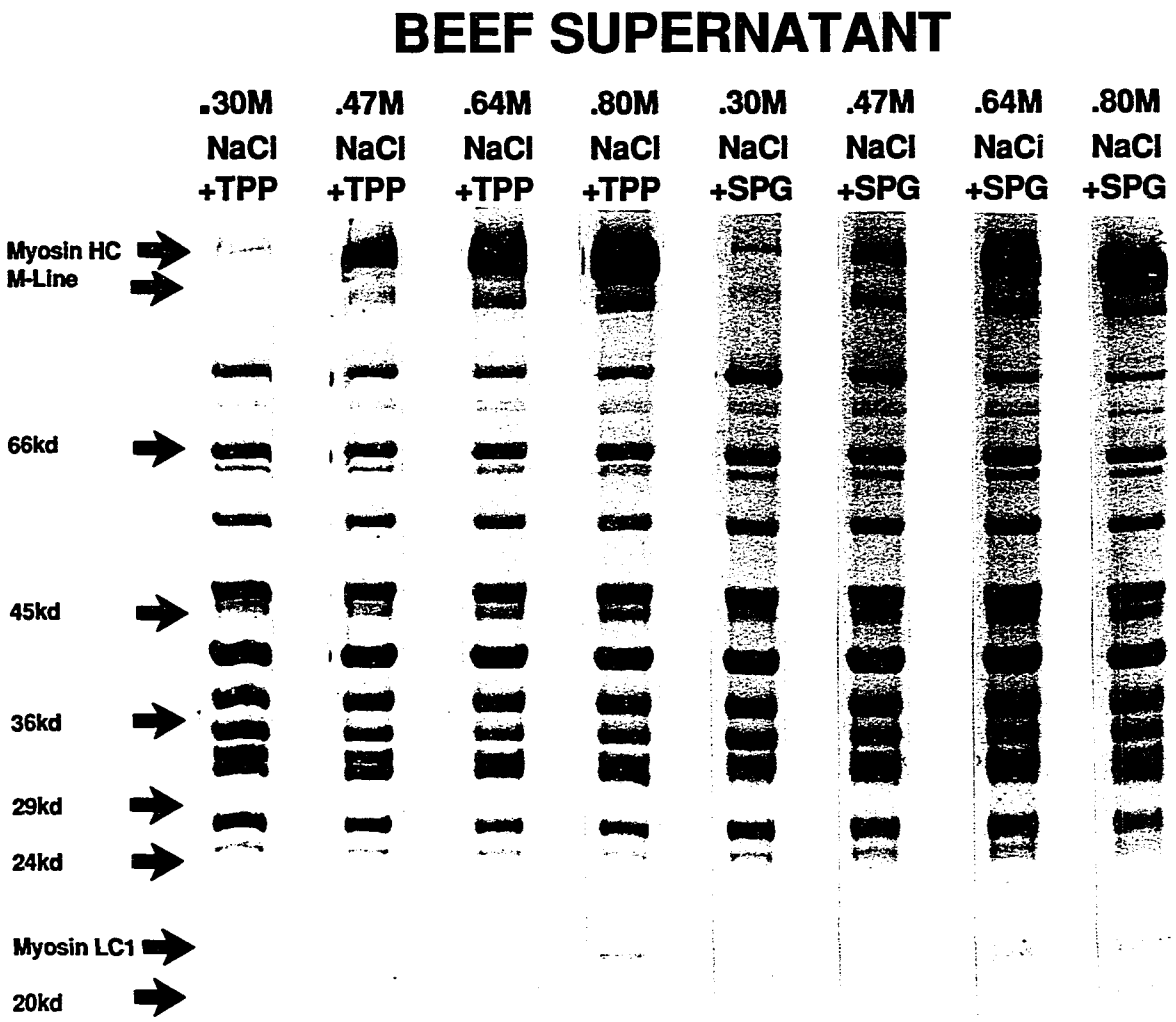

BEEF SUPERNATANT
.30M NaCl +TPP
.47M NaCl +TPP
.64M NaCl +TPP
.80M NaCl +TPP
.30M NaCl +SPG
.47M NaCl +SPG
.64M NaCl +SPG
.80M NaCl +SPG
Myosin HC
M-Line
66kd
45kd
36kd
29kd
24kd
Myosin LC1
20kd

Fig. 15 - 12% SDS-gels (Coomassie blue-stained) of purified myofibrillar proteins from beef sternomandibularis muscle after treatment with various NaCl and NaCl+10mM PP solutions

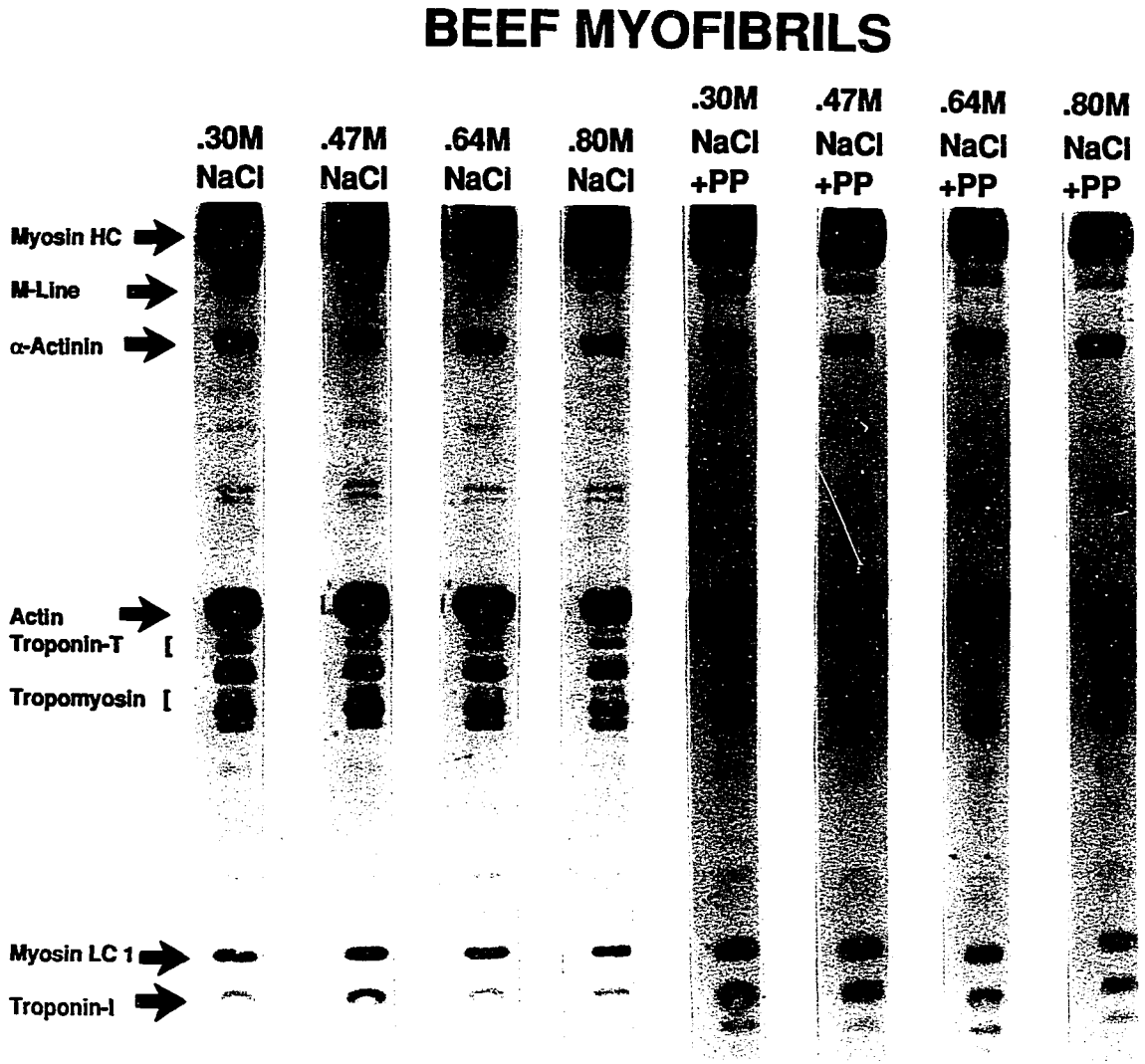
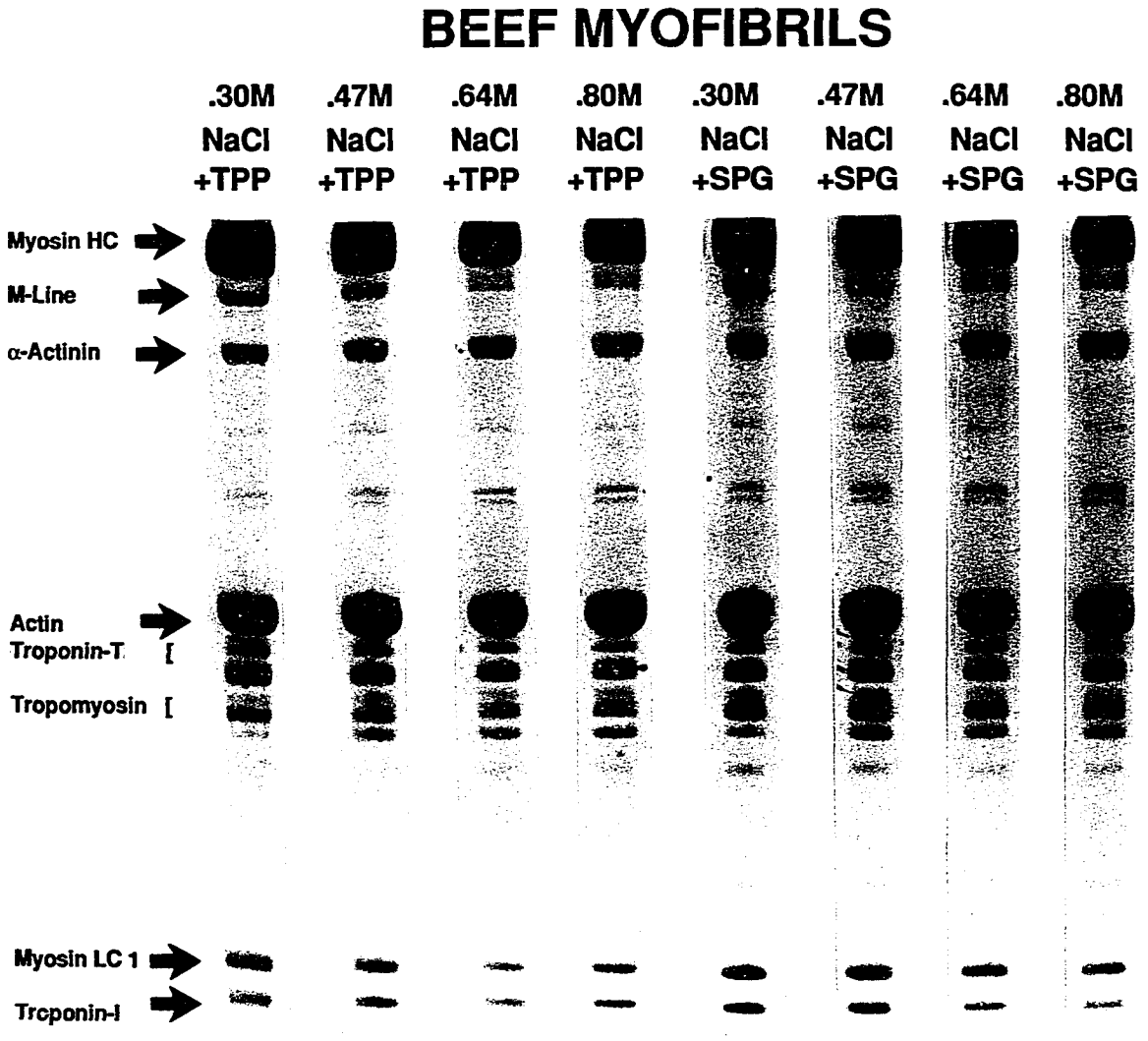

BEEF MYOFIBRILS
.30M NaCl
.47M NaCl
.64M NaCl
.80M NaCl
.30M NaCl +PP
.47M NaCl +PP
.64M NaCl +PP
.80M NaCl +PP
Myosin HC
M-Line
α-Actinin
Actin
Troponin-T
Tropomyosin
Myosin LC 1
Troponin-I

Fig. 16 - 12% SDS-gels (Coomassie blue-stained) of purified myofibrillar proteins from beef sternomandibularis muscle after treatment with various NaCl+10mM TPP and NaCl+10mM SPG solutions


BEEF MYOFIBRILS
.30M NaCl +TPP
.47M NaCl +TPP
.64M NaCl +TPP
.80M NaCl +TPP
.30M NaCl +SPG
.47M NaCl +SPG
.64M NaCl +SPG
.80M NaCl +SPG
Myosin HC
M-Line
α-Actinin
Actin
Troponin-T
Tropomyosin
Myosin LC 1
Troponin-I

REFERENCES

LaSalle, F., Robson, R. M., Lusby, M. L., Parrish, F. C., Jr., Stromer, M. H., and Huiatt, T. W. 1983. Localization of titin in bovine skeletal muscle by immunofluorescence and immunoelectron microscope labeling. J. Cell Biol. 97:258a.

Lewis, D. F. 1981. The use of microscopy to explain the behavior of foodstuffs - a review of work carried out at the Leatherhead Food Research Association. Scanning Electron Microsc. III:391-404.

Lewis, D. F., Groves, K. H. M., and Holgate, J. H. 1986. Action of polyphosphates in meat products. Food Microstructure. 5:53-62.

Offer, G., and Trinick, J. 1983. On the mechanism of water holding in meat: The swelling and shrinking of myofibrils. Meat Sci. 8:245-281.

Paterson, B. C., Parrish, F. C., Jr., and Stromer, M. H. 1987. Response of beef sternomandibularis muscle to various salt and pyrophosphate treatments. J. Food Sci. Submitted.

Shults, G. W., and Wierbicki, E. 1973. Effects of sodium chloride and condensed phosphates on the water-binding capacity, pH and swelling of chicken muscle. J. Food Sci. 38:991-994.

Shults, G. W., Russell, D. R., and Wierbicki, E. 1972. Effect of condensed phosphates on pH, swelling and water-holding capacity of beef. J. Food Sci. 37:860-864.

Trout, G. R., and Schmidt, G. R. 1984. The effect of phosphate type, salt concentration and method of preparation on the binding in restructured beef rolls. J. Food Sci. 49:687-694.

Trout, G. R., and Schmidt, G. R. 1986a. Effect of chain length and concentration on the degree of dissociation of phosphates used in food products. J. Agric. Food Chem. 34:41-45.

Trout, G. R., and Schmidt, G. R. 1986b. Effect of phosphates on the functional properties of restructured beef rolls: the role of pH, ionic strength, and

phosphate type. J. Food Sci. 51:1416-1423.

Voyle, C. A., Jolley, P.D., and Offer, G. W. 1984. The effect of salt and pyrophosphate on the structure of meat. Food Microstruct. 3:113-126.

Wang, K., and Williamson, C. L. 1980. Identification of an N_2-line protein of striated muscle. Proc. Natl. Acad. Sci. USA 77:3254-3258.

Wang, K., McClure, J., and Tu, A. 1979. Titin: major myofibrillar components of striated muscle. Proc. Natl. Acad. Sci. USA 76:3698-3702.

Wang, K., Ramierez-Mitchell, R., and Palter, D. 1984. Titin is an extraordinarily long and flexible myofibrillar protein. Proc. Natl. Acad. Sci. USA 81:3685-3689.

Journal Paper No. J- of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project 2711.

SUMMARY AND CONCLUSIONS

Despite considerable research, reviewed extensively by Hamm (1960, 1970), Ranken (1976) and Trout and Schmidt (1983), questions still remain concerning the effects of NaCl and phosphates on the WHC of meat and meat products. Offer and Trinick (1983) suggested that when phosphate, particularly PP, was combined with NaCl, transverse structural restraints such as Z-lines, M-lines and actin-myosin crossbridges were disrupted because NaCl+PP solutions were effective at extracting myofibrillar protein. Voyle et al. (1984) reported similar findings and associated this increased protein extraction due to the presence of NaCl+PP with increased meat WHC values. Lewis et al. (1986) treated pork and beef muscles with NaCl solutions containing different kinds of phosphates. Although they observed some extraction of protein from muscles due to NaCl and phospahtes, Lewis et al. (1986) attributed increased WHC to the phosphates ability to increase the pH of the meat system. The effort of these studies was to examine closely the effects of NaCl and phosphates on beef muscle.

Studies with NaCl and NaCl+10mM PP

Offer and Trinick (1983) and Voyle et al. (1984) showed that the addition of 10mM PP to NaCl solutions increased

protein extraction, myofibril swelling and WHC above solutions containing only NaCl. This study confirmed those results by combining phase-contrast microscopy, SDS-PAGE and WHC tests to show the effects of NaCl and NaCl+10mM PP on beef muscle. The results showed that: (1) higher NaCl concentrations increased beef myofibril swelling, increased myofibrillar protein extraction and improved beef WHC; (2) the addition of 10mM PP to NaCl solutions decreased the NaCl concentration required for maximal myofibril swelling; (3) the presence of 10mM PP also increased protein extraction, especially of titin and myosin, and substantially improved beef muscle WHC; (4) the extraction of titin seemed to be an important event in regulating increased myofibril swelling and increased WHC.

Studies with NaCl and NaCl+10mM PP, 10mM TPP and 10mM SPG

Shults et al. (1972) and Trout and Schmidt (1984, 1986a) concluded that different phosphates affect muscle tissue differently but, as a generalization, the different phosphates increased functional properties in the following order: PP > TPP > SPG. The results of this study confirm that earlier work and show that: (1) as NaCl concentration increased, within each treatment group, myofibril swelling, myofibrillar protein extraction and beef WHC increased; (2) solutions containing NaCl+10mM PP or 10mM TPP were more

effective at increasing myofibril swelling than NaCl+10mM SPG which was only slightly more effective than NaCl alone; (3) certain solutions containing NaCl+10mM PP extracted almost all of the myofibril A-bands starting from the A-/I-band junctions while NaCl+10mM TPP, NaCl+10mM SPG and solutions containing only NaCl extracted less myofibrillar protein and A-/I-band junction generally remained resistant to extraction by these solutions; (4) solutions containing NaCl+10mM PP or 10mM TPP were more effective at increasing beef muscle WHC than NaCl+10mM SPG which was only marginally more effective than solutions containing only NaCl; (5) the extraction of a critical amount of myofibrillar protein or a critical type of myofibrillar protein seemed to be necessary to produce maximal myofibril swelling and WHC, and (6) the cytoskeletal protein titin may be the critical protein of which extraction was necessary to increase myofibril swelling and beef muscle WHC.

LITERATURE CITED

Anonymous. 1982. Meat and poultry products. Phosphate and sodium hydroxide. Fed. Reg. 47(49):10779-10785.

Awad, M. K. 1968. Hydrolysis of polyphosphates added to meat. Master's Thesis. The University of Alberta, Edmonton, Alberta, Canada.

Baldwin, T. T., and deMan, J. M. 1968. Mineral composition of meat treated with citrate and phosphates. Can. Inst. Food Technol. J. 1(4):164-169.

Bendall, J. R. 1954. The swelling effect of phosphates on lean meat. J. Sci. Food and Agric. 5:468-475.

Bendall, J. R. 1973. Postmortem changes in muscle. In Bourne, G. H. (Ed.). The Structure and Function of Muscle. Vol. 2. Academic Press, Inc., New York.

Carlsen, F., Fuchs, F., and Knappeis, G. G. 1965. Contractility and ultrastructure in glycerol-extracted muscle fibers. II. Ultrastructure in resting and shortened fibers. J. Cell Biol. 27:35-46.

Craig, R., and Offer, G. 1976. The isolation of C-protein in rabbit skeletal muscle. Proc. R. Soc., London, B, 192:451-461.

Ellinger, R. H. 1972. Phosphates as food ingredients. In The CRC Handbook of Food Additives. Vol. 1. CRC Press, Cleveland, OH.

Ellinger, R. H. 1977. Phosphates as food ingredients. Pp. 48-73. In The CRC Handbook of Food Additives. Vol. 1. 2nd ed. CRC Press, Cleveland, OH.

Everson, C. W. 1985. Non-meat ingredients. Proc. Ann. Sausage and Processed Meat Short Course. Iowa State University Extension Service, Ames, IA.

Forrest, J. C., Aberle, E. D., Hedrick, H. B., Judge, M. D., and Merkle, R. A. 1975. Properties of fresh meat. Pp. 174-189. In Schweigert, B. S. (Ed.). Principles of Meat Science. W. H. Freeman and Co., San Francisco, CA.

Franzini-Armstrong, C. 1970. Details of the I-band structure as revealed by the localization of ferritin.

Tissue Cell 2:327-338.

Fukazawa, T., Hashimoto, T., and Yasui, T. 1961. The relationship between the components of myofibrillar protein and the effect of various phosphates that influence the binding quality of sausage. J. Food Sci. 26:550-555.

Gillett, T. A., Brown, C. L., Leutzinger, R. L., Cassidy, R. D., and Simon, S. 1978. Tensile strength of processed meats determined by an objective Instron technique. J. Food Sci. 43:1121-1124.

Goll, D. E., Robson, R. M., and Stromer, M. H. 1984. Skeletal muscle. Pp. 548-580. In Swenson, J. M. (Ed.). Duke's Physiology of Domestic Animals. 10th edition. Cornell University Press, Ithaca, New York.

Grabowski, J., and Hamm, R. 1979. Protein solubility and water binding under the conditions obtained in Bruhwurst mixtures. Fleischwirtschaft 59:1166-1169.

Granicher, D., and Portzehl, H. 1964. The influence of magnesium and calcium pyrophosphate chelates of free magnesium ions, free calcium ions, and free pyrophosphate ions on the dissociation of actomyosin in solution. Biochim. Biophys. Acta 86:567-570.

Greaser, M. L., Wang, S. M., and Lemanski, L. F. 1981. New myofibrillar proteins. Proc. Recip. Meat Conf. 34:12-16.

Grove, B. K., and Eppenberger, H. M. 1983. Appearance of myomesin and M-protein during chicken muscle development. J. Cell Biol. 97:551a. (Abstr.).

Hamm, R. 1960. Biochemistry of meat hydration. Adv. Food Res. 10:355-463.

Hamm, R. 1970. Interactions betweeen phosphates and meat proteins. Pp. 65-82. In Demann, J. M., and Melnychyn, P. (Eds.). Symposium: Phosphates in Food Processing. AVI Publishing Co., Westport, CT.

Hamm, R. 1975. Water binding capacity of meat. In Cole, D. D. A., and Lawrie, R. (Eds.). Meat. Butterworths, London.

Hamm, R. 1977. Changes of muscle proteins during the heating of meat. In Hoyem, T., and Kvale, O. (Eds.).

Physical, Chemical and Biological Changes in Food Caused by Thermal Processing. Appl. Sci. Pub., Ltd., London.

Hamm, R. 1981. Postmortem changes in muscle affecting the quality of comminuted meat products. In Lawrie, R. (Ed.). Developments in Meat Science - 2. Appl. Sci. Pub., Ltd., London.

Hamm, R., and Neraal, R. 1977a. On the enzymatic breakdown of tripolyphosphate and diphosphate in comminuted meat. VII. Influence of sodium chloride on the triphosphatase and diphosphatase activity in bovine muscle. Z. Lebensm. - Unters. - Forsch. 164:34-37.

Hamm, R., and Neraal, R. 1977b. On the enzymatic breakdown of tripolyphoosphate and diphosphate in comminuted meat. X. Influence of substrate concentration. Z. Lebensm. - Unters. - Forsch. 164-98-100.

Hamm, R., and Neraal, R. 1977c. On the enzymatic breakdown of tripolyphosphate and diphosphate in comminuted meat. XII. Influence of the breakdown of tripolyphosphate and diphosphate on the water-holding-capacity of meat. Z. Lebensm. - Unters.- Forsch. 164:243-246.

Hamm, R., and van Hoof, J. 1974. Influence of sodium chloride on the breakdown and colloid-chemical effect of ATP in postrigor ground beef muscle. Z. Lebensm. - Unters. - Forsch. 156:87-90.

Hellendoorn, E. W. 1962. Water-binding capacity of meat as affected by phosphates. Food Technol. 16:119-124.

Honikel, K. O., Fischer, C. H., Hamid, A., and Hamm, R. 1981. Influence of postmortem changes in bovine muscle on the water holding capacity of beef: Postmortem storage of muscle at 20°C. J. Food Sci. 46:1-6.

Huiatt, T. W., Robson, R. M., Arakawa, N., and Stromer, M. H. 1980. Desmin from avian smooth muscle: purification and partial characterization. J. Biol. Chem. 255:6981-6989.

Huxley, H. E. 1958. The contraction of muscle. Scientific American 213, No. 6:18-27.

Huxley, H. E. 1972. Molecular basis of contraction in cross striated muscle. Pp. 301-364. In Bourne, G. H. (Ed.). The Structure and Function of Muscle. Vol. 1.

2nd ed. Academic Press, Inc., New York, New York.

Huxley, H. E., and Hanson, J. 1954. Changes in cross striations of muscle during contraction and stretch and their interpretation. Nature 173:973-976.

Inklaar, P. A. 1967. Interaction between polyphosphates and meat. J. Food Sci. 32:525-526.

Ishiorishi, M., Samejime, K., and Yasui, T. 1979. Heat - induced gelation of myosin: Factors of pH and salt concentrations. J. Food Sci. 44:1280-1284.

Kiely, B., and Martonosi, A. 1968. Kinetics and substrate binding of myosin adenosine triphosphatase. J. Biol. Chem. 243:2273-2278.

Kimura, S., and Maruyama, K. 1983. Preparation of native connectin from chicken breast muscle. J. Biochem. 94:2083-2085.

Knight, P. J., and Parsons, N. J. 1984. Variable response of beef myofibrils to salt solutions. Proc. 30th Europ. Meet. Meat Res. Workers. 30:118-119.

Laki, K. 1971. Actin. Pp. 97-133. In Laki, K. (Ed.). Contractile proteins and muscle. Marcel Dekker, Inc., New York, New York.

LaSalle, F., Robson, R. M., Lusby, M. L., Parrish, F. C., Jr., Stromer, M. H., and Huiatt, T. W. 1983. Localization of titin in bovine skeletal muscle by immunofluorescence and immunoelectron microscopy. J. Cell Biol. 97:285a. (Abstr.).

Lawrie, R. A. 1983. Aspects of the biochemistry of meat. Int. J. Biochem. 3:233-242.

Lewis, D. F., Groves, K. H. M., and Holgate, J. H. 1986. Action of polyphosphates in meat products. Food Microstructure. 5:53-62.

Locker, R. H. 1982. A new theory of tenderness in meat, based on gap filaments. Proc. Recip. Meat Conf. 35:92-100.

Locker, R. H., and Leet, N. G. 1975. Histology of highly stretched beef muscle. I. The fine structure of grossly stretched fibers. J. Ultrastruct. Res. 52:64-75.

Locker, R. H., and Leet, N. G. 1976. Histology of highly stretched beef muscle. II. Further evidence on the nature and location of gap filaments. J. Ultrastruct. Res. 55:157-172.

Lusby, M. L., Ridpath, J. F., Parrish, F. C., Jr., and Robson, R. M. 1983. Effect of postmortem storage on degradation of the myofibrillar protein titin in bovine longissimus muscle. J. Food Sci. 48:1787-1790.

Lyons, J. W., and Siebenthal, C. D. 1966. On the binding of condensed phosphates by proteins. Biochim. Biophys. Acta 120:174-176.

Mahon, J. H. 1961. Tripolyphosphate-salt synergism and its effect on cured meat volume. Proc. 13th Meat Ind. Res. Conf. 13:59-67.

Mak, A. S., Smillie, L. B., and Stewart, G. R. 1980. A comparison of the amino acid sequences of rabbit skeletal alpha- and beta-tropomyosins. J. Biol. Chem. 255:3647-3655.

Maruyama, K. 1976. Connectin, an elastic protein of myofibrils. J. Biochem. 80:405-407.

Maruyama, K. 1985. Myofibrillar and cytoskeletal proteins of vertebrate striated muscle. Pp. 25-50. In Lawrie, R. (Ed.). Developments in Meat Science. Elsevier Applied Sci. Pub., London and New York.

Maruyama, K., Kimura, S., Kuroda, M., and Handa, M. 1977. connectin, an elastic protein of muscle. Its abundance in cardiac myofibrils. J. Biochem. 82:347-350.

Maruyama, K., Kimura, S., Toyota, N., and Ohashi, K. 1980. Connectin, an elastic protein of muscle. Pp. 33-42. In Perry, A. D. and Creamer, K. L. (Eds.). Fibrous Proteins: Scientific, Industrial, and Medical Aspects. Vol. 2. Academic Press, London.

Maruyama, K., Kimura, S., Ohashi, K., and Kuwano, Y. 1981. Connectin, an elastic protein of muscle. Identification of "titin" with connectin. J. Biochem. 89:701-709.

Moos, C. 1981. Fluorescence microscope study of the binding of added C-protein to skeletal muscle myofibrils. J. Cell Biol. 90:25-31.

Naus, K. M., Kitagawa, S., and Gergely, J. 1969. Pyrophosphate binding to and adensoine triphosphate activity of myosin and its proteolytic fragments. J. Biol. Chem. 244:755-765.

Neraal, R., and Hamm, R. 1977a. On the enzymatic breakdown of tripolyphosphate and diphosphate in minced meat. V. Change in the diphosphatase activity of muscle postmortem. Z. Lebensm. - Unters. - Forsch. 163:208-212.

Neraal, R., and Hamm, R. 1977b. On the enzymatic breakdown of tripolyphosphate and diphosphate in comminuted meat. VIII. Influence of divalent cations on the tripolyphosphatase activity of muscle tissue. Z. Lebensm. - Unters. - Forsch. 164:38-40.

Offer, G., and Trinick, J. 1983. On the mechanism of water holding in meat: The swelling and shrinking of myofibrils. Meat Sci. 8:245-281.

O'Shea, J. M., Robson, R. M., Hartzer, M. K., Huiatt, T. W., Rathbun, W. E., and Stromer, M. H. 1981. Purification of desmin from adult mammalian skeletal muscle. Biochem. J. 195:345-356.

Page, S. G. 1968. Fine structure of tortoise skeletal muscle. J. Physiol. 197:709-715.

Paterson, B. C., and Parrish, F. C., Jr. 1986. A sensory panel and chemical analysis of certain beef chuck muscles. J. Food Sci. 51:876-879.

Puolanne, E., and Matikkala, M. 1980. Effect of pH value on water-binding capacity of cooked sausage. Fleischwirtschaft 60:1233-1235.

Puolanne, E., and Ruusunen, M. 1980. Effect of salt and phosphate on water-binding capacity in cooked sausage. Fleischwirtschaft 60:1359-1361.

Ranken, M. D. 1976. The water holding capacity of meat and its control. Chem. Ind. 1976:1052-1057.

Regenstein, J. M., and Stamm, J. R. 1979. Factors affecting the sodium chloride extractability of muscle proteins from chicken breast, trout white and lobster tail muscles. J. Food Biochem. 3:191-204.

Ridpath, J. F., Robson, R. M., Huiatt, T. W., Trenkle, A. H., and Lusby, M. L. 1982. Localization and rate of accumulation of nebulin in embryonic chick cardiac muscle cell cultures. J Cell Biol. 95:361a. (Abstr.).

Robson, R. M., and Huiatt, T. W. 1983. Roles of the cytoskeletal proteins desmin, titin and nebulin in muscle. Proc. Recip. Meat Conf. 36:116-124.

Robson, R. M., Goll, D. E., Arakawa, N., and Stromer, M. H. 1970. Purification and properties of alpha-actinin from rabbit skeletal muscle. Biochim. Biophys. Acta 200:296-318.

Romans, J. R., Jones, K. W., Costello, W. J., Carlson, C. W., and Ziegler, P. T. 1985. Meat as a food. In The Meat We Eat. 12th Edition. The Interstate Printers and Publishers, Inc., Danville, Illinois.

Schut, J. 1976. Meat Emulsions. Pp. 385-458. In Friberg, S. (Ed.). Food Emulsions. Marcel Dekker, Inc., New York.

Sherman, P. 1961. The water binding capacity of fresh pork. I. The influence of sodium chloride, pyrophosphates and polyphosphates on water absorption. Food Technol. 15:79-97.

Shults, G. W., Russell, D. R., and Wierbicki, E. 1972. Effect of condensed phosphates on pH, swelling and water holding capacity of beef. J. Food Sci. 37:860-864.

Sjostrand, F. S. 1962. The connections between A- and I-band filaments in striated frog muscle. J. Ultrastruct. Res. 7:225-246.

Starr, K., and Offer, G. 1978. The interaction of C-protein with heavy meromyosin and subfragment-2. Biochem. J. 171:813-816.

Sutton, A. H. 1973. The hydrolysis of sodium triphosphate in cod and beef muscle. J. Food Technol. 8:185-195.

Suzuki, A., Goll, D. E., Singh, I., Allen, R. E., Robson, R. M., and Stromer, M. H. 1976. Some properties of purified skeletal muscle alpha-actinin. J. Biol. Chem. 251:6860-6870.

Szent-Gyorgi, A. G. 1960. Proteins of the myofibril. Pp. 1-49. In Bourne, G. H. (Ed.). The structure and function of muscle. Vol. 2. Academic Press, Inc., New York.

Trinick, J. A., and Lowey, S. 1977. M-protein from chicken pectoralis muscle: Isolation and characterization. J. Mol. Biol. 113:343-368.

Trinick, J., Knight, P., and Whiting, A. 1984. Purification and properties of native titin. J. Mol. Biol. 180:331-356.

Trout, G. R., and Schmidt, G. R. 1983. Utilization of phosphates in processed meat products. Proc. Recip. Meat Conf. 36:24-27.

Trout, G. R., and Schmidt, G. R. 1984. Effect of phosphate type and concentration, salt level and method of preparation on binding in restructured beef rolls. J. Food Sci. 49:687-694.

Trout, G. R., and Schmidt, G. R. 1986a. Effect of phosphates on the functional properties of restructured beef rolls: The role of pH, ionic strength, and phosphate type. J. Food Sci. 51:1416-1423.

Trout, G. R., and Schmidt, G. R. 1986b. Effect of chain length and concentration on the degree of dissociation of phosphates used in food products. J. Agric. Food Chem. 34:41-46.

Vandegrift, V., and Evans, R. R. 1981. Polyphosphate binding interactions with bovine serum albumin in protein - polyphosphate precipitates. J. Agric. Food Chem. 29:536-539.

Van Wazer, J. R. 1970. Chemistry of the Phosphates and Condensed Phosphates. In Deman, J., and Melnychyn, P. (Eds.). Symposium: Phosphates in Food Processing. AVI Publishing Co., Westport, CT.

Voyle, C. A., Jolley, P. D., and Offer, G. W. 1984. The effect of salt and pyrophosphate on the structure of meat. Food Microstructure 3:113-126.

Wang, K. 1982. Purification of titin and nebulin. Methods in Enzymol. 85:264-273.

Wang, K. 1984. Sarcomere - associated cytoskeletal lattices in striated muscle, review and hypothesis. Pp. 315-369. In Shay, J. W. (Ed.). Cell and Muscle Motility. Vol. 6. Plenum Press, New York.

Wang, K., and Williamson, C. L. 1980. Identification of an N_2-line protein of striated muscle. Proc. Natl. Acad. Sci. USA 77:3254-3258.

Wang, K., McClure, J., and Tu, A. 1979. Titin: major myofibrillar components of striated muscle. Proc. Natl. Acad. Sci. USA 76:3689-3702.

Wang, K., Ramirez-Mitchell, R., and Palter, D. 1984. Titin is an extraordinarily long flexible, and slender myofibrillar protein. Proc. Natl. Acad. Sci. USA 81:3685-3689.

Wierbicki, E., Cahill, V. R., and Deatherage, F. E. 1957. Effects of added sodium chloride, potassium chloride, calcium chloride, magnesium chloride and citric acid on meat shrinkage at 70°C and of added sodium chloride on drip losses after freezing and thawing. Food Technol. 11:74-76.

Wierbicki, E., Tiede, M. G., and Burrell, R. C. 1962. Determination of meat swelling as a method for investigating the water-binding capacity of muscle protein with low water-holding forces. I. The methodology. Fleischwirtschaft 14:948-951.

Yamaguchi, M., Robson, R. M., and Stromer, M. H. 1983. Evidence for actin involvement in cardiac Z-lines and Z-line analogues. J. Cell Biol. 96:335:442.

Yasui, T., Sakanishe, M., and Hashimoto, Y. 1964a. Effect of inorganic polyphosphates on the solubility and extractability of myosin B. J. Agric. Food Chem. 12:392-398.

Yasui, T., Fukazawa, T., Takahashi, K., Sakanishi, M., and Hashimoto, Y. 1964b. Specific interactions of inorganic polyphosphates with myosin B. J. Agric. Food Chem. 12:399-404.

Yates, L. D., and Greaser, M. L. 1983. Quantitative determination of myosin and actin in rabbit skeletal muscle. J. Mol. Biol. 168:123-141.

Zeece, M. G., Robson, R. M., Lusby, M. L., and Parrish, F. C., Jr. 1986. Effect of calcium activated protease (CAF) on bovine myofibrils under different conditions of pH and temperature. J. Food Sci. 51:797-803.

ACKNOWLEDGEMENTS

I want to express my greatest appreciation to my major professor, Dr. F. C. Parrish, Jr., for his assistance, advice and encouragement in planning and conducting this research and for his guidance throughout my graduate studies at Iowa State University. I would also like to thank Dr. Joe Sebranek, Dr. Dennis Olson, Dr. Homer Walker, Dr. Marvin Stromer and Dr. Ted Huiatt for serving on my committee and for reviewing this thesis.

I extend a special thanks to fellow graduate students Mike Lesiak, Mark Kruel and Jeff Paxhia for their help and advice. I would expecially like to thank Kathy Schnieder for her technical assistance and Dr. Parrish's lab technicians, Nan Fowler, Kent Pulfer and Kris Tressler for their efforts with my research.

I wish to express my deepest appreciation to my wife, Kim, and my parents, Bill and Eileen, for their encouragement, support and love during my years of graduate work at South Dakota State University and Iowa State University.